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Modulación del transcriptoma inmune de trucha por la proteína no viriónica (NV) del Virus de la Hemorragia Septicémica Viral (VHSV). Nuevos hallazgos que correlacionan la secuencia de NV con su función.

Immune transcriptome modulation in trout by the non-virion (NV) protein from Viral Haemorrhagic Septicaemia Virus (VHSV). New insights correlating NV sequence with function.

MEMORIA PARA OPTAR AL GRADO DE DOCTORA PRESENTADA POR

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CERTIFICAN:

Que la Tesis Doctoral titulada **“Modulación del transcriptoma inmune de trucha por la proteína no viriónica (NV) del Virus de la Hemorragia Septicémica Viral (VHSV). Nuevos hallazgos que correlacionan la secuencia de NV con su función.”,** cuyo título en inglés es **“Immune transcriptome modulation in trout by the non-virion (NV) protein from Viral Haemorrhagic Septicaemia Virus (VHSV). New insights correlating NV sequence with function”,** ha sido realizada por la licenciada Dª. Blanca Chinchilla Rodríguez, en el Departamento de Biotecnología del INIA, bajo la dirección conjunta de los que suscriben, y estimamos que cumple con la normativa exigida para su presentación como Compendio de Publicaciones para optar al grado de Doctor por la Universidad Complutense de Madrid (UCM).

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Madrid, 13 de septiembre de 2018

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To my beloved family

“La différence entre un rêve et un project, c’est une date”

(Walt Disney)

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El género *Novirhabdovirus* pertenece a familia *Rhabdoviridae,* y está formado por cuatro especies (VHSV, IHNV, HIRRV y SHRV) que infectan exclusivamente peces y se caracterizan por la presencia de un gen adicional, que codifica para una proteína no viriónica (NV) que da nombre al género. El virus de la septicemia hemorrágica viral (VHSV) tiene gran importancia para la acuicultura mundial debido al amplio y creciente rango de hospedadores que infecta, siendo la trucha arcoíris uno de los principales hospedadores del continente europeo, pero también porque su presencia es considerada de declaración obligatoria para la Organización Mundial de Sanidad Animal (OIE).

El gen NV no tiene similitud con ningún otro conocido hasta el momento y las proteínas NV presentan una alta homología dentro de una misma especie pero muy reducida entre especies diferentes. A pesar de ello, las proteínas NV de IHNV y de VHSV comparten una función similar, siendo ambas prescindibles pero necesarias para una eficiente replicación viral *in vitro* y esenciales para la patogenicidad *in vivo* de los novirhabdovirus. El efecto de ambas proteínas NV se ha relacionado *in vitro* e *in vivo* con una menor respuesta antiviral celular. Sin embargo, hasta ahora no se conocían en detalle los genes y mecanismos de respuesta inmune modulados por la NV, y tampoco la implicación de la estructura en su función. En base a lo expuesto, esta tesis planteó como principales objetivos: 1) caracterizar por primera vez el transcriptoma inmune de trucha inducido por la NV recombinante de VHSV, sin la interferencia de otras proteínas del virus, y 2) delimitar las regiones implicadas en la función, y explicar la aportación que tienen algunos aminoácidos de estas regiones en la misma.

Para ello, previamente se obtuvo la proteína recombinante NV de VHSV (07.71) y se inoculó intraperitonealmente en truchas y, posteriormente, se analizó el conjunto de RNAs procedentes del bazo y riñón cefálico de cada trucha. El perfil transcriptómico se determinó mediante un microarray enriquecido en genes de respuesta inmune, diseñado y validado en el laboratorio. Los resultados mostraron que la gran mayoría de los genes estudiados tenían una regulación negativa y su implicación era desconocida hasta el momento. Entre los genes regulados a la baja se encuentran interferones, factores de necrosis tumoral, genes inducidos por VHSV (VIG), interleuquinas, citoquinas, complemento, factores de transcripción y genes de procesamiento y presentación antigénica. Además, entre los genes que participan en mayor

número de vías de activación (genes multipath) se encuentran factores de transcripción como *stat1*, *jun*, y *atf1*; y genes relacionados con interferones y factores de necrosis tumoral. Esto explicaría por qué hay una activación deficiente de las vías de señalización implicadas, que a su vez conllevaría la baja expresión de otros genes de respuesta inmune relacionados. Así, la proteína NV inhibe la respuesta inmune innata y adaptativa humoral y celular, interfiriendo con la respuesta antiviral temprana del hospedador.

Por otro lado, el mapeo funcional de la NV se llevó a cabo con 8 fragmentos de la proteína NV de VHSV (F1 a F8) midiendo la inhibición de la expresión de transcritos de *mx e il8* en células de pez cebra (ZF4) transfectadas. El fragmento N-terminal más completo de la NV (F1) inhibió *mx e il8* de manera similar a la NV completa, mientras que el fragmento C-terminal F2 perdió la función original de la NV. Otros fragmentos N-terminales más pequeños conservaron también la función, aunque incluyeran parte de la secuencia C-terminal, sugiriendo que la parte N-terminal es más importante que la C-terminal para la función. Para llevar a cabo una caracterización funcional más completa de la NV, también generamos 11 variantes de la proteína en las posiciones 28, 31, y de la 33 a la 41. Mientras algunas variantes conservaron o perdieron ligeramente la función, las variantes NVD36A, NVR39A y NVD41A incrementaron significativamente su capacidad de inhibición de *mx* e *il8* en células ZF4. Todas las proteínas NV completas, nativas y variantes, presentaron la misma localización citosólica perinuclear de aspecto punteado. Además, los fragmentos N-terminal de la NV también presentaron la misma localización citosólica mientras que el fragmento C-terminal, no funcional (F2), estaba difusamente distribuido en la célula. Esto sugiere que la localización está correlacionada con la función, y la función con la parte N-terminal de NV. Recientemente se ha caracterizado un ligando de NV, PPM1Bb, capaz de defosforilar TBK1 e interferir posteriormente con la respuesta de RIG-I. Las variantes NVD36A, NVR39A, y NVD41A, por su parte, serían más efectivas en la estabilización de PPM1Bb y posterior defosforilación de TBK1, resultando en una mayor downregulación de *mx* e *il8*. Así, los resultados del perfil transcriptómico de trucha inducidos por la NV permiten conocer mejor el modelo de infección por VHSV y los procesos de estimulación-inhibición de vías de señalización de la respuesta inmune frente a rhabdovirus.

Finalmente, el desarrollo de una técnica de detección semi-automática de anticuerpos neutralizantes frente a VHSV en pequeños volúmenes de plasma permitiría su aplicación al estudio de la efectividad de las vacunas frente al virus, particularmente aquellas basadas en la deleción de la NV.

Palabras clave: Trucha, VHSV, NV, transcriptoma, inhibición de la respuesta inmune.

SUMMARY

Immune transcriptome modulation in trout by the non-virion (NV) protein from Viral Haemorrhagic Septicaemia Virus (VHSV). New insights correlating the NV sequence with function.

Novirhabdovirus genus belongs to the family *Rhabdoviridae,* and it consists of four species (VHSV, IHNV, HIRR and SHRV) that exclusively infect fish and are characterized by the presence of an additional gene encoding a non-virion (NV) protein that gives name to the genus. Viral haemorrhagic septicaemia virus (VHSV) is of great importance for global aquaculture due to the wide and growing range of hosts it infects, being rainbow trout one of the main hosts of the European continent, but also because its presence is considered of mandatory notification to the World Organization for Animal Health (OIE).

The NV gene has no similarity to any other known gene so far and NV proteins show high homology intra-species but very low inter-species. In spite of this, NV proteins of IHNV and VHSV share a similar function; they both are dispensable but necessary for an efficient viral replication *in vitro* and essential for *in vivo* pathogenicity of the novirhabdoviruses. The effect of both NV proteins has been linked *in vitro* and *in vivo* with a lower cell antiviral response. However, genes and immune response mechanisms modulated by NV were not known in detail until now, nor was the relationship between the structure and function. On the basis of the above, this thesis proposed as main objectives: 1) to characterize for the first time the immune-related transcriptome of trout induced by recombinant NV of VHSV, without the interference of other viral proteins, and 2) to define the regions involved in the function, and to explain the contribution of some amino acids from these regions to the function.

For that purpose, recombinant VHSV NV protein (07.71) was previously obtained and intraperitoneally injected into trouts and subsequently the RNA pool of the spleen and head kidney of each trout was analysed. The transcriptome profile was determined by using an enriched microarray of immune response genes, designed and validated by the laboratory. The results showed that the great majority of the genes studied were down-regulated and their involvement was unknown until now. Among these down-regulated genes are interferons, tumor necrosis factors, genes induced by VHSV (VIG), interleukins, cytokines, complement, transcription factors and genes for antigenic processing and presentation. Moreover, among the genes involved in a greater number of activation pathways (multipath genes) are transcription factors such as *stat1*, *jun*, and *atf1*; and genes related to interferons and tumor necrosis factors. This would explain why there is a poor activation of the signaling pathways involved that would in turn lead to low expression of other immune-related genes. Thus, NV protein inhibits both humoral and cellular processes of innate and adaptive immune responses, interfering with early antiviral response of the host.

On the other hand, the NV functional mapping was performed with 8 fragments of the VHSV NV protein (F1 to F8) by measuring the inhibition of the expression of *mx* and *il8* transcripts in transfected zebrafish (ZF4) cells. The most complete N-terminal fragment of NV (F1) inhibited *mx* and *il8* similarly to whole NV whereas the C-terminal fragment F2 lost NV original function. Other smaller N-terminal fragments also retained function, even if they included part of the C-terminal sequence, suggesting that the N-terminal part is more important than the C-terminal for the function. To carry out a more complete functional characterization of NV, we also generated 11 variants of the protein in positions 28, 31, and 33 to 41. While some variants conserved or slightly lost function, variants NVD36A, NVR39A and NVD41A increased significantly its ability to inhibit *mx* and *il8* in ZF4 cells. All complete NV proteins, native and variants, presented the same cytosolic location with a perinuclear dotted appearance. In addition, the N-terminal fragments of NV also showed the same cytosolic location while the non-functional C-terminal fragment (F2) was diffusely distributed within the cell. This suggests that the location is correlated with the function, and in turn, the function with the N-terminal part of NV. Recently, it has been described a ligand of NV, PPM1Bb, capable of dephosphorylating TBK1 and subsequently interfering with the response of RIG-I. The variants NVD36A, NVR39A, and NVD41A, for their part, would be more effective in stabilizing PPM1Bb and further dephosphorylation of TBK1, thus resulting in a greater downregulation of *mx* and *il8*. Thus, the results of the trout transcriptomic profile induced by NV allow for better understanding of VHSV infection model and the processes of stimulation-inhibition of immune signaling pathways against rhabdoviruses.

Finally, the development of a semi-automatic assay to detect anti-VHSV neutralizing antibodies in low volumes of plasma blood samples could allow its application in future studies on vaccine effectiveness, particularly those based on NV deletion.

Keywords: Trout, VHSV, NV, transcriptome, inhibition of immune response.

ANNEXES

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LIST OF ABBREVIATIONS

atf1	-Activating Transcription Factor 1
casp	-Caspases
CPE	-Cytopathic Effect
cRNA	-complementary RNA (antigenome)
DIVA	-Differentiating between Infected and Vaccinated Animals
DNA	-Deoxyribonucleic acid
EPC	-*Epithelioma Papulosum Cyprinid* cells.
GFP	-Green Fluorescence Protein
HIRRV	-Hirame rhabdovirus
ICTV	-International Committee for Taxonomy of Viruses
IFN	-interferon
IHNV	-Infectious Haematopoietic Necrosis Virus
IRF	-Interferon-Regulatory Factors
ISG	-IFN-stimulated genes
kb	-kilobases
kDa	-kilodaltons
MAbs	-Monoclonal Antibodies
MAVS	-Mitochondrial Antiviral Signaling protein
MHC	-Major Histocompatibility Complex (MHC)
mx	-Orthomyxovirus resistance gene
NFkB	-Nuclear factor-kB (NF-kB)
NLS	-Nuclear Location Signal
NV	-Non-Virion gene or protein
OIE	-World Organisation for Animal Health
ORF	-Open Reading Frame
PAMP	-Pathogen-Associated Molecular Patterns
pfu	-Plaque-forming units
PPM1Bb	-Protein phosphatase, Mg^{2+}/Mn^{2+}-dependent, 1Bb
PRR	-Pattern Recognition Receptors
RdRp	-RNA-dependent-RNA polymerase complex
RIG-I	-Retinoic acid inducible gene I
RNA	-Ribonucleic acid
RNP	-Ribonucleoprotein complex
RPS	-Relative Percent Survival
RTG-2	-Rainbow Trout Gonad cells
RV	-Rabies Virus
SHRV	-Snakehead Rhabdovirus
ssRNA	-single-stranded RNA
STAT	-Signal transducer and activator of transcription
SVCV	-Spring Viraemia of Carp Virus
TBK1	-Traf family member-associated NF-κB activator (TANK)-binding kinase 1
TLR	-Toll-like receptor
TNFα	-Tumor Necrosis Factor α
TRAF	-TNF receptor-associated factor
VHSV	-Viral Haemorrhagic Septicaemia Virus
vRNA	-viral RNA
VSV	-Vesicular Stomatitis Virus
WT, wt	-Wild-Type
ZF4	-Zebrafish cell line

LIST OF PUBLICATIONS

This thesis is based on the following publications:

Paper I

Transcriptome analysis of rainbow trout in response to non-virion (NV) protein of viral haemorrhagic septicaemia virus (VHSV).
Chinchilla B., Encinas P., Estepa A., Coll J.M., Gomez-Casado E.

Applied Microbiology and Biotechnology. 2015 Feb; 99 (4): 1827-43.
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Paper II

Identification of the functional regions of the viral haemorrhagic septicaemia virus (VHSV) NV protein: Variants that improve function.
Chinchilla B., Gomez-Casado E.

Fish & Shellfish Immunology. 2017 Nov; 70: 343-350.
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Paper III

Optimization of fixed-permeabilized cell monolayers for high throughput micro-neutralizing antibody assays: Application to the zebrafish/viral hemorrhagic septicemia virus (vhsv) model.
Chinchilla B., Encinas P., Estepa A., Coll J.M., Gomez-Casado E.

Journal of Virological Methods. 2013 Nov; 193(2): 627-632.
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I. INTRODUCTION

A. VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS) DISEASE

1. Importance of VHS disease

Aquaculture broadly refers to the farming of aquatic organisms, including finfish, shellfish, crustaceans and plants, primarily for human consumption, and has been practiced since antiquity. However, it was not until the mid-20th century that intensive, industrial-scale aquaculture became viable, and recent decades have witnessed a dramatic expansion such that it is currently the fastest growing food producing sector globally [1]. In a world where more than 800 million people continue to suffer from chronic malnourishment [2], and where the global population is expected to grow and reach 9.6 billion people by 2050, aquaculture plays an increasingly critical role in global food security. Four decades ago, aquaculture accounted for only 7% of all fish consumed globally, with the vast majority being produced by the capture sector [3]. However, a milestone was reached in 2014, when for the first time, aquaculture accounted for the majority of fish consumed globally [4].

The development of large-scale intensive aquaculture has been associated with a dramatic increase in severe disease outbreaks caused by a diverse range of pathogens, including parasites, viruses and bacteria [5]. These disease outbreaks can be catastrophic for the industry and are especially relevant for the World Organisation for Animal Health (OIE) notifiable pathogen species, the presence of which may incur the destruction of stock or a temporary ban on trading between states [6].

Viral haemorrhagic septicaemia (VHS) is one of the most serious viral diseases of the aquaculture industry worldwide. A notable characteristic and part of the reason for the importance of this virus in so many countries in the world is the very large and continually increasing number of fish species that can be infected with clinical consequences and high mortality rates [7]. This is in direct contrast to the very similar virus, IHNV (most recently renamed as *Salmonid novirhabdovirus*), the impacts of which are largely restricted to salmonids [8].

Historically, European rainbow trout production has been threatened by viral haemorrhagic septicaemia (VHS), which annually leads to substantial economic losses in the trout farming industry. The annual losses of rainbow trout in Europe since the first appearance of VHSV on fish farms have been estimated to exceed 40 million pounds per year [9]; however, the true economic cost of VHS is not well characterized. These losses can be a direct consequence of fish mortality or an indirect consequence of disease control measures.

Due to its high mortality and large economic impact, VHS is classified as a notifiable disease by the World Organization for Animal Health (OIE) [10] and the European Union (Council Directive 2006/88/EC) [11]. For all the reasons mentioned above, VHS remains one of the most important diseases of finfish worldwide.

2. Host factors and disease pattern

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. However, VHSV is not known to infect fish eggs, which can carry the virus externally without being affected [12, 13]. On the other hand, several factors influence susceptibility to VHS disease. In rainbow trout there is genetic variability for susceptibility [5], and the age of the fish appears to be of some importance. In general, the disease is more abundant in populations of young, not previously infected fish (the younger the fish the higher the susceptibility) [14].

The virus usually causes a severe disease with mortality rates as high as 100% in fry, but ranges from 5% to 90% in older rainbow trout. During acute VHS infection, fish do exhibit external disease signs such as skin darkening, exophthalmia (bulging eyes), internal and external haemorrhages on the skin, gills, eyes, and internal organs (mainly in kidney and liver), ascites (bloated abdomens) and/or erratic swimming behaviour **(Figure 1)**. However, varied forms of disease have been described for VHS (acute, chronic and nervous) in rainbow trout (*Oncorhynchus mykiss*) [15], and a persistent infection/asymptomatic carrier state for VHSV has also been reported being often associated with neural tissues. Therefore, it has been proposed that the brain offers the potential for virus to persist and escape immunodetection, and this may have significant implications in the maintenance of viral reservoirs in wild fish [16-21].

VHS is commonly a cool- or cold-water disease that is most prevalent and readily transmitted when fish congregate during the spring spawning season at temperatures of 9–12°C [22-24]. Its viral particles can live for up to 13 days in the water, depending upon temperature [25], and are transmitted from fish to fish (horizontal transmission) through contact with the urine and reproductive fluids of other infected fish and also via shed mucus [22, 26, 27], through virus-contaminated water or objects (ballast water and boating, fishing tackle) [27, 28] and diverse vectors including amphipod crustaceans (*Diporeia spp*) [29], leeches [30], freshwater turtles [31], and piscivorous birds [32, 33]. However, the movement of infected fish, for example baitfish [34-36], represents a risk factor for introducing VHSV to new areas.

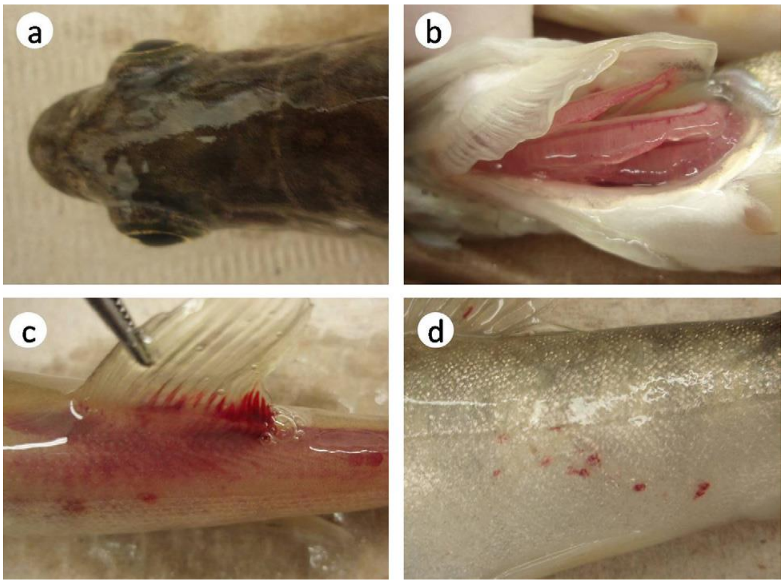

Figure 1. Clinical signs of VHSV infection. (a) Lake trout (*Salvelinus namaycush*) showing moderate exophthalmia, (b) severe gill pallor (c), hemorrhaging at the base of the dorsal fin of a juvenile muskellunge (d), and petechial hemorrhages in the epidermis of a splake (*Salvelinus namaycush · S. fontinalis*). Figure obtained from Kim and Faisal 2011 [27].

With regard to viral entry, the main route is suggested to be the skin and/or gills [37], being the fin bases more important than the gills in rainbow trout [38-40]. Recently, vaccination studies showed that the virus can have an entry route through the gut epithelial layer [41, 42] and this oral transmission route has been demonstrated for rainbow trout [43] and tiger muskellunge (*Esox lucius × E. masquinongy*) [34] through food-borne, water-borne and cohabitation infections.

3. VHS etiology and history

The disease was first recognized as early as 1938 when Danish rainbow trout farms became stricken with what Schäperclaus described as “infectious kidney swelling and liver degeneration” [44]. Even though a viral etiology was early suspected by Schäperclaus because the disease was transmitted using bacteria-free filtrates of tissue homogenates from affected fish, the viral etiology was not confirmed until 1963, when Jensen isolated the virus on a rainbow trout gonad cell line (RTG-2) and promptly described it as “Egtved” virus [45]. The disease was later renamed as Viral Haemorrhagic Septicaemia by the Office of International Epizooties (currently known as the World Animal Health Organization) and this terminology remained until 2000 when the International Committee for Taxonomy of Viruses (ICTV) changed the name of the virus into VHSV [46].

Until the late 1980s, VHSV was thought to be strictly a disease of farmed rainbow trout (*Oncorhynchus mykiss*) of freshwater in Europe; and further typing of those European strains showed that they all were within genotype Ia [47]. However, the detection of VHSV in returning adult Chinook and Coho salmon in North America in 1988, later defined as genotype IVa, confirmed the expansion of VHSV range to that continent [48, 49].

The following years (1990-1999), new more VHSV isolations occurred in North America, causing fatal disease in fish from marine and brackish environments of Washington and Alaska, and also other fish species were found affected (e.g.: Pacific cod, Pacific herring, etc.) [26], providing evidence that VHSV was more widespread in the marine environment than previously thought. Such discoveries in the coastal waters of Alaska and the Pacific Northwest led to a study conducted by Hedrick et al. 2003 [50], which revealed the presence of VHSV in new hosts (sardine, mackerel and 2 species of smelt) and extended the geographic range (Oregon and California, USA).

During this same time frame, VHSV continued to devastate freshwater and marine waters in Europe and simultaneous reports of VHSV outbreaks were described. Moreover, the North American VHSV isolations ignited further testing of marine fish populations in European waters. Later identification of VHSV (genotypes I to III) in a vast number of marine fish species in Europe [51] raised the suspicion that VHSV was of marine origin and investigations into the source of the trout-pathogenic European genotype Ia isolate showed that it likely emerged from a marine source some 60 years earlier [47]. In addition to Europe and North America, VHSV has also been isolated in Japan from wild Japanese flounder (*Paralichthys olivaceus*) in 1999 and in Korea [52-54]. Based on the information gathered from all over the world, by the late 1990s, a consensus started building that VHSV primarily affects marine fish and that VHSV may have a marine origin [55].

The expansive and highly adaptable nature of VHSV was clearly underestimated after being isolated from adult muskellunge sampled from Lake St. Clair, Michigan between 2003 and 2005 [56], at which time no other rhabdoviruses were known to infect any fish species in the Great Lakes Basin. Currently, the VHSV-related events in the Great Lakes Basin have emphasized the need to study the origin of VHSV and its ability to devastate fishes in both the freshwater and marine environments and the path it takes to spread.

More recently VHSV was isolated from rainbow trout in the United Kingdom [57]. While this resurgence of VHSV in Europe is not surprising, the isolation reported by Stone et al. 2008 [57] demonstrated the first VHSV isolation in the United Kingdom (UK) obtained from a freshwater species in a freshwater environment. The investigators attributed the outbreak to a nearby stream that may have been harboring the virus for a long period of time.

Taken together, these results emphasized the fact that while legislation, biosecurity, and sound management practices can greatly reduce the spread of VHSV, the virus' covert nature enables it to continue expanding into new geographic locations. The re-emergence of VHSV was also experienced in Norway in seawater-farmed rainbow trout [58]. The authors concluded that the outbreak was most likely a result of viral transmission from the marine environment. Both isolations have thus confirmed VHSV's capacity to emerge and resurge in the northern hemisphere.

4. Control and prevention: vaccination

"Prevention is better than cure" is a well-known expression in human as well as veterinary medicine. Preventive measures are especially important in aquaculture where the number of individuals at risk is high, pathogenic microorganisms may be transmitted through water (which disinfection is difficult or even impossible) and where chemotherapy is a challenge from practical, technical, economic and environmental points of view [59].

Prevention and control of infectious disease by fish vaccination is becoming increasingly important as a part of aquaculture biosecurity. Vaccination has a positive effect on fish farming and the aquaculture industry by reducing morbidity, mortality and the costs of therapy, and by improving product quality. Vaccination may also improve animal welfare by reducing the suffering of diseased fish. Moreover, vaccinated animals have a reduced risk of disease development and even non-vaccinated animals may be protected due to herd immunity [60]. Finally, prophylaxis based on stimulation of the immune system also contributes to a sustainable aquaculture with low use of antibiotics [59].

Although research on vaccine development for VHS has been ongoing for more than four decades, a commercial vaccine against VHS is not yet available. Several candidate vaccines have been tested to control this disease, among them live attenuated vaccines [61-63], inactivated vaccines [64-66] and recombinant protein vaccines [67-69] but with limited efficacy or compromised safety aspects and also difficulties associated with the requirement for large-scale production and mass delivery system for vaccination [65, 70, 71] (see **Table 1** for a comparative analysis).

A recent alternative approach to engineer new and possible safer live attenuated vaccines, including knockouts, is based on reverse genetics (see NV role section of thesis) [72-75]. Moreover, recombinant viruses recovered by reverse genetics can be engineered as “DIVA” (differentiating between infected and vaccinated animals) vaccines, thus allowing easy discrimination between vaccinated and naturally-infected fish in the field [76]. However, additional work is needed to improve the safety of such vaccines [77].

By contrast, DNA vaccines have shown promising results. DNA vaccines containing the G gene of VHSV were first reported to be protective in 1998 [78]. Since then, several VHSV DNA vaccines have been developed and tested in numerous experimental trials and they have been shown to be highly efficacious in inducing specific and protective immunity under a wide range of conditions and in several marine and freshwater fish species (for review see [77, 79]). In addition to the European freshwater VHSV DNA vaccines, there is now a DNA vaccine against a marine strain of VHSV that has shown high efficacy in flounder [80, 81]. The success of these vaccines led to the licensing and commercialization of a DNA vaccine against a related novirhabdovirus, IHNV, for Atlantic salmon (APEX-IHN; Novartis Animal Health Canada Inc.) in Canada in 2005, with no outbreaks reported since [82]. Nevertheless, safety considerations have not allowed yet their commercialization in Europe (DNA-vaccinated fish are considered as genetically modified organisms) and a mass delivery system for vaccination of fish fry is required. However, their efficacy varies with the relatedness of the challenge strain to the strain used to create the vaccine [83]. The majority of publications using these vaccines describe homologous challenges, but it has also been tested for efficacy against heterologous VHSV strains [84], providing significant protection but with reduced efficacy compared to the homologous virus strain (54 to 78 relative percent survival (RPS) compared to 77 to 100 RPS). Moreover, dual vaccination assays with both VHSV and IHNV DNA vaccines have been shown to induce neutralizing antibodies specific to both viruses and protect fish against coinfection [85-87]. Although DNA vaccines seem to be effective, well tolerated and safe for fish, several aspects need to be improved, such as the route of delivery to allow mass immunization and safety concerns (DNA persistence, environmental release, etc.) [77].

Since no cross-protection exists between VHSV and IHNV, an efficient vaccine against VHSV is still required. Meanwhile, control of VHSV in the absence of any treatment (and taking into account the difficulties in treating fish infected) and commercial vaccines, is achieved by prevention of introduction and eradication by stamping-out in case of disease outbreaks. This is the case of Denmark, which through a permanent surveillance and control program, has been declared VHS-free and no new outbreak was recorded for more than two years [88].

Table 1. Advantages and disadvantages of VHSV vaccines (reviewed from [71, 77]).

VHS Vaccines				
Types		***Description***	***Advantages***	***Disadvantages***
Replicating vaccines ***(Live-attenuated vaccines)***		*Attenuation is obtained through multiple passages of VHSV on cell culture, neutralization escape mutants and thermoresistant viruses. Currently, attenuation is also obtained by reverse genetics.*	High levels of protection by bath immersion and oral delivery. Attenuated viruses by reverse genetics allow discrimination between vaccinated and naturally-infected fish (DIVA) and they could be used as safe live-attenuated vaccines.	Differences in fish disease susceptibility and safety problems (residual virulence was observed in most cases).
Non-replicating vaccines	***Inactivated vaccines***	*Inactivation of whole virus can be easily achieved either by chemical (e.g. β-propiolactone) or physical (e.g.UV) treatment.*	Efficient in inducing long-term protection when delivered by intraperitoneal injection and do not require the use of adjuvants.	Protection was not sufficient by bath immersion challenge and, for this reason, are not suitable for mass delivery in young fish. An additional concern is the loss of immunogenicity during the inactivation process.
	Subunit vaccines	*VHSV subunit vaccines are based on the recombinant G protein, the unique target of neutralizing antibodies, expressed in prokaryotic and eukaryotic cells.*	Detection of neutralizing antibodies in sera.	Vaccination with recombinant G protein did not elicit good protection, despite the induction of neutralizing antibodies, and low levels of protection were only achieved when administrated by intraperitoneal injection in combination with an adjuvant. Due to its cost of production and route of delivery, does not appear to be suitable for mass delivery to fish fry.
	Synthetic peptide vaccines	*Peptide vaccines use short amino acid sequences as antigens. Are based on known antigenic epitopes of the VHSV G protein.*	Attenuated *Aeromonas salmonicida* was tested as a vector for the expression of VHSV G protein fragments. Fish immunized by bath immersion or spray was moderately protected against homologous challenge.	Synthetic peptide vaccine alternatives were also poorly immunogenic.
DNA vaccines		*DNA vaccination relies on the use of a gene or genes encoding protective antigens, instead of the antigens themselves. These vaccines are based on G gene of VHSV.*	They have been shown to be highly efficacious in inducing specific and protective immunity under a wide range of conditions and in several fish species. DNA vaccines also protect against heterologous strains but with reduced efficacy compared to the homologous.	Their efficacy varies with the relatedness of the challenge strain to the strain used to create the vaccine. Several aspects concerning technical (route of delivery) and safety problems (fish DNA persistence) need to be addressed.

B. VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV)

1. Nomenclature and classification

The International Committee on Taxonomy of Viruses (ICTV) [89] assigned the *Novirhabdovirus* as a new genus, which belongs to the family *Rhabdoviridae* [90] within the order *Mononegavirales*. *Novirhabdovirus* genus includes four members that are exclusively fish pathogens: Viral haemorrhagic septicaemia virus (VHSV), Infectious haematopoietic necrosis virus (IHNV), Hirame rhabdovirus (HIRRV) and Snakehead rhabdovirus (SHRV); and the prototype species of the novirhabdovirus is IHNV [91].

VHSV (initially named as Egtved virus) is the aetiological agent of the viral haemorrhagic septicaemia disease, and its name was recently changed to *Piscine novirhabdovirus* (formerly *Oncorhynchus 2 novirhabdovirus*), while IHNV has been renamed as *Salmonid novirhabdovirus*. These names were suggested by Kurath and Kuhn to the ICTV *Rhabdoviridae* Study Group [92, 93] in a move to highlight the exceptionally wide host range of VHSV, compared to the more narrow host range of other genus members such as IHNV, which is limited to salmonid fish. However, despite the new names approved, these viruses are still known as VHSV and IHNV and this nomenclature is used for convenience in this thesis.

In contrast to other mammal and fish rhabdoviruses (e. g.: Vesiculovirus, SVCV), members of the genus *Novirhabdovirus* are distinguished by the presence of a unique additional gene, called the 'non-virion' or 'NV' gene, which gives the name to the genus **(Figures 2 and 3)**, and has no significant similarity with any other viral gene [46]. In addition, comparison of nucleotide and amino acid sequences of the structural proteins also supported the classification of the novirhabdoviruses into a separate genus **(Figure 2)** [94].

2. The genome organisation, replication and virion properties

The typical novirhabdoviral genome contains six open reading frames (ORF) and encodes five basic structural proteins (namely in the 3' to 5'order): nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G), and large RNA-dependent RNA polymerase (L); and a non-structural protein ('non-virion' or NV), located between G and L proteins **(Figure 4A)** [95-97]. Additionally, all the ORF of VHSV are separated by untranslated sequences, known as gene junctions, whereas the untranslated regions at the 3'- and 5'-end are known as the 'leader' and 'trailer', respectively [89, 96]. The gene order of VHSV is 3'-leader-N-

P-M-G-NV-L-trailer-5' as other members of the *Novirhabdovirus* genus **(Figures 3 and 4A)** and the genes are sequentially transcribed [96].

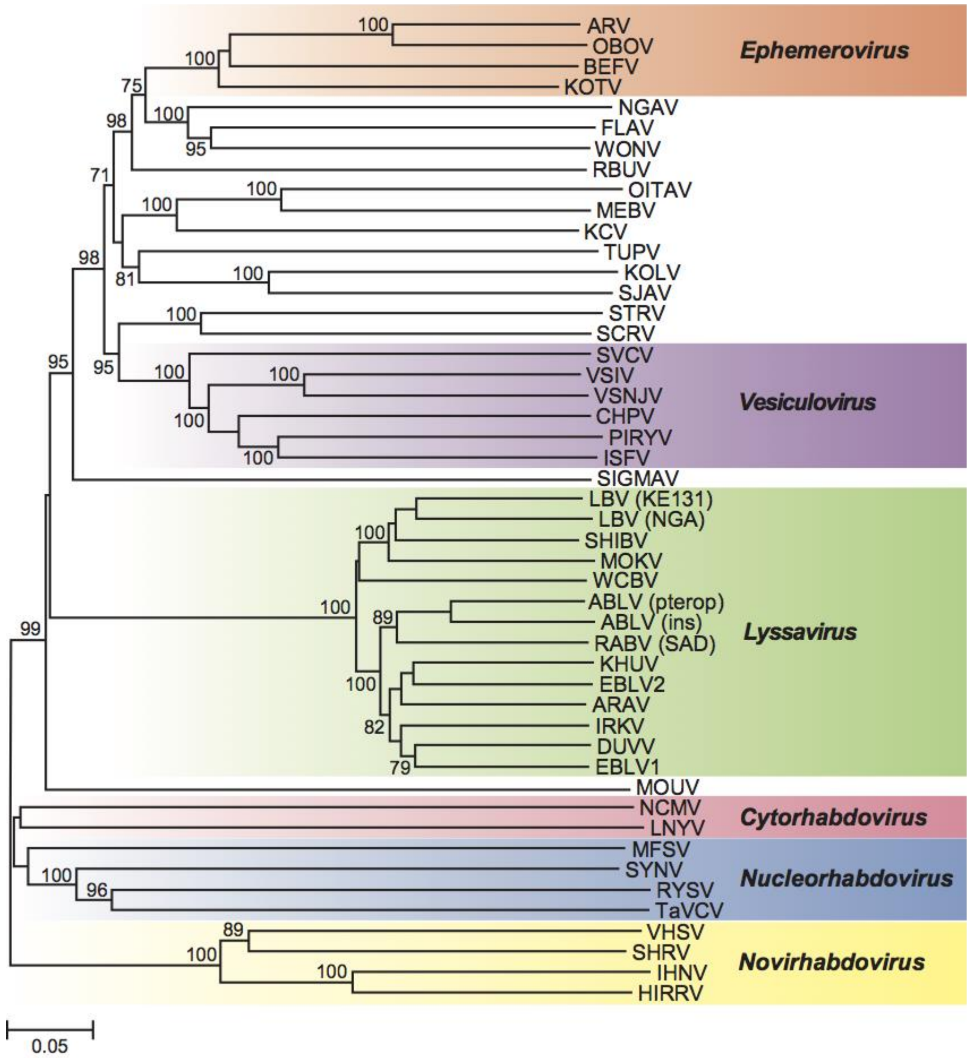

Figure 2. Phylogenetic tree of viruses in the *Rhabdoviridae* based on alignment of nucleoprotein gene sequences. The tree was generated by the neighbor-joining method using 1000 bootstrap replicates (obtained from [98]).

All the genes of VHSV start with a conserved gene-start sequence (-CGUG-), followed by an ORF and a conserved gene-end sequence (A/GUCUAU/ACU^7) with 7 uracil (U) residues, which are polyadenylation signal for polymerase when it transcribes a gene. After this poly (A) signal, there are two conserved intergenic di-nucleotides (G or A), which are untranscribed and act as spacers between the two genes [99]. The viral polymerase transcribes the genome responding to these specific gene-start and gene-end sequences and skips these two nucleotides to start transcribing the next gene and to yield a series of discrete monocistronic mRNAs [100]. The mRNAs are not produced in equimolar amounts; rather, their relative abundance decreases with distance from a single 3′-proximal polymerase entry site (such that N > P > M > G > NV > L) [101-104], being promoter-proximal genes transcribed in greater abundance than distal genes [105].

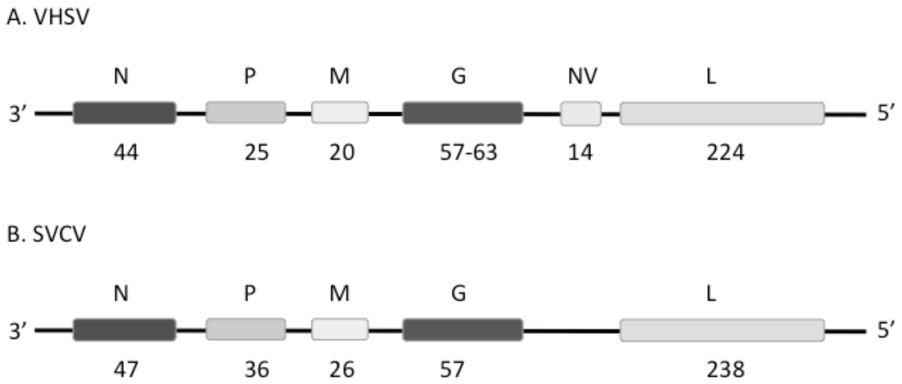

Figure 3. Comparison of the genome structure among two fish rhabdovirus: (A) a member of the genus *Novirhabdovirus*, viral haemorrhagic septicemia virus (VHSV); and (B) a typical member of the genus *Vesiculovirus*, spring viremia of carp virus (SVCV). Predicted SVCV MW are based on [106] and for VHSV on [96].

Virions are bullet-shaped (approximately 70 nm in width and 180 nm in length) **(Figure 4B)** and contain a simple negative-sense, single-stranded RNA (ssRNA) genome of approximately 11-11.2 kb and possess an envelope that consists of a lipid bilayer membrane **(Figure 4C)** [96].

3. The structural proteins: characteristics and functions

As reviewed above, VHSV virions have five structural proteins designated N, P, M, G and L. The G and N genes of VHSV are less genetically variable than the NV gene [24], whereas the P gene and the M gene appear to be even more highly conserved [107]. The N gene's more rapid evolutionary rate may enhance ability of the virus to evade the host's induction of interferon and chemokines, as hypothesized for rabies [108].

In general, the primary functions and basic characteristics of the five structural proteins of novirhabdoviruses have been assumed to be the same as demonstrated for the homologous proteins of mammalian rhabdoviruses vesicular stomatitis (VSV) and rabies viruses (RV), and are conserved across all rhabdoviruses. However, some functions and characteristics may differ among them or has not been specifically proven in most cases (see **Table 2** for summary of their characteristics and functions).

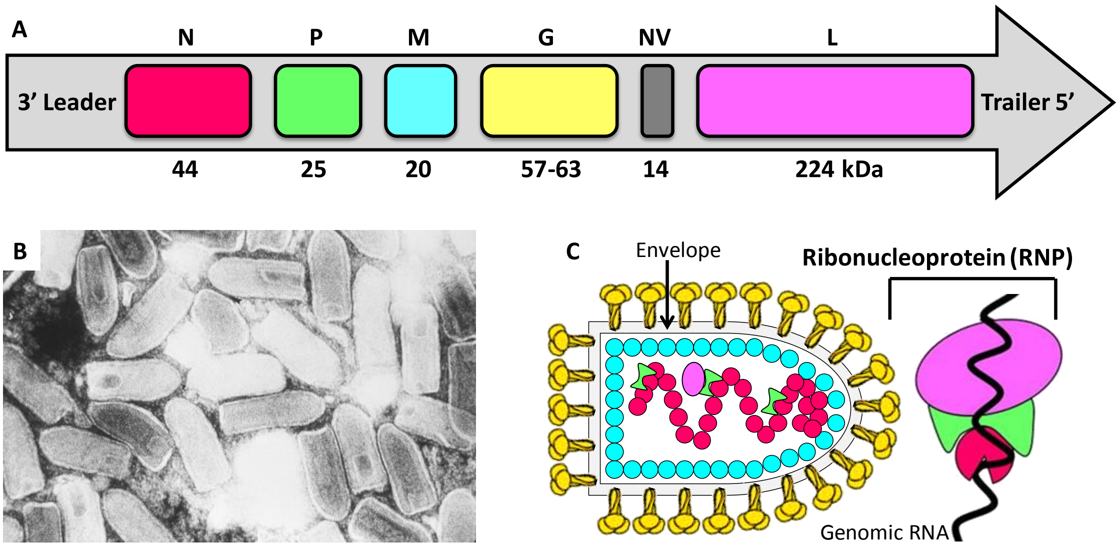

Figure 4. Organization of the VHSV genome (A), morphology (B) and schematic representation of structural components of rhabdoviruses (C). (A) The gene order of VHSV is 3'-leader-N-P-M-G-NV-L-trailer-5'. The arrow indicates the sense of gene transcription from the 3' to 5' end of the template virus RNA and in decreasing molar abundances [95]. Genome organization and predicted molecular weights (MW) in kilodaltons of a typical member of the genus *Novirhabdovirus*, Viral haemorrhagic septicaemia virus (VHSV). Predicted VHSV molecular weights are based on [109]. (B) Electron micrograph depicts the bullet-shaped morphology of virions from the *Rhabdoviridae* family (image obtained from CDC, 1981).

The structural proteins consist of two major functional units: a **ribonucleoprotein (RNP) complex** unit composed of N, P, and L for transcription and replication of genomic and anti-genomic viral RNA, and **envelope components** composed of G and M proteins to allow RNP in and out of the host cell [96].

Ribonucleoprotein (RNP) complex

The nucleoprotein (N) of VHSV spans 404 amino acids and has a molecular mass of 44 kDa approximately. This is the earliest expressed and most abundant protein produced by the virus during VHSV infection. As a constituent of the RNP unit, the N-protein encapsidates the genome (negative-strand RNA, vRNA) protecting it from nucleases and forms part of the ribonucleoprotein core structure of virion, which serves as the template for viral transcription (viral RNA to mRNA), for replication (cRNA to viral RNA) and viral genome copying **(Figure 5)**. This encapsidated genomic RNA is also associated with the phosphoprotein (P) and polymerase protein (L), which are involved in viral protein synthesis and replication [110].

Table 2. Summary of the characteristics, functions, and molecular weights (MW) of the five structural proteins of VHSV based on [96].

VHSV Structural Proteins		*MW*	*Characteristics and functions*
G	**Glycoprotein**	**57-63 kDa**	Surface antigen (which forms trimers) that facilitates virus **attachment and entry** into the host cell and elicits the production of **neutralizing antibodies.** It has been used for vaccination experiments and also for VHSV genotyping/typing.
M	**Matrix protein**	**20 kDa**	Apart from its critical role in **viral assembly and budding**, it is also implicated in host **gene suppression** during infection.
N	**Nucleoprotein**	**44 kDa**	N is bound to the viral RNA (protecting it from nucleases) and modulates the balance between genome **transcription and replication processes**.
P	**Phosphoprotein**	**25 kDa**	P serves as a cofactor for the viral polymerase allowing **viral replication and inhibits activation of interferon response** in the host cells (i.e., blocks antiviral responses).
L	**Polymerase**	**224 kDa**	Guides **viral replication and transcription** and it also plays a role in **host specificity and temperature**.

The phosphoprotein (P) spans 231 amino acids and has a molecular mass of 25 kDa. Its role is to facilitate viral replication and inhibit the activation of interferon response in the host cells. The interaction of N protein with phosphoprotein (P) abolishes any non-specific RNA binding, and prevents phosphorylation. The phosphoprotein has a highly variable N-terminal half and a conserved C-terminal half [99]. Phosphoprotein, which is a non-catalytic polymerase-associated protein, has a putative conserved domain for binding with L protein to be a complete RNA-dependent-RNA polymerase (RdRp) complex [111, 112]. Based on vesicular stomatitis virus (VSV, *Rhabdoviridae*), the P has three domains: an N-terminal domain to bind with the L protein, a central oligomerization domain, and a C-terminal domain to bind with the N-RNA template [113, 114].

The polymerase gene encodes a protein (L protein) of 1,986 amino acids with a predicted molecular weight of approximately 224 kDa. The RNA polymerase activity of the large protein (L) guides viral transcription and replication [107], and potentially plays a role in growth of the virus at different temperatures [107].

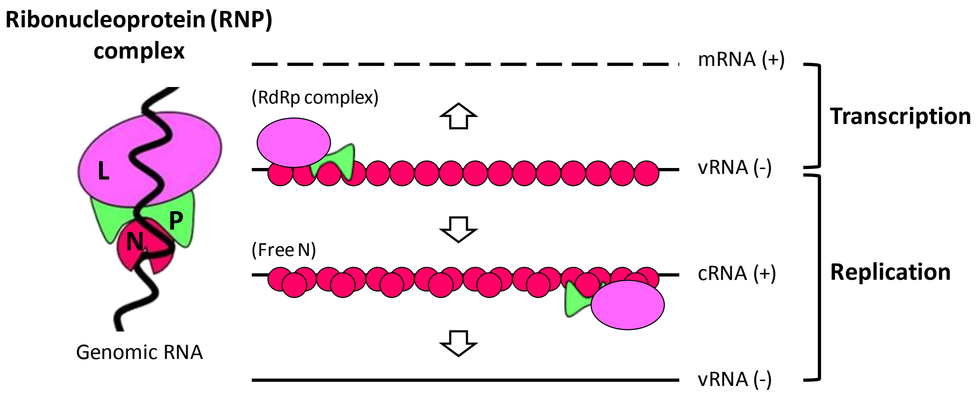

Figure 5. Transcription and replication cycle of negative-strand RNA viruses. In the absence of free nucleocapsid (N) protein, the RNA-dependent-RNA polymerase (RdRp) complex transcribes the genome to make mRNA at the early infection stage. After translation of the N proteins, the complex uses the highly N-coated complementary RNA (cRNA) genome as a copy to make more viral RNA (vRNA) and to produce more viral proteins, especially RNPs [115] (Figure modified from [116]).

Envelope components

The viral envelope contains the major surface antigen, the glycoprotein (G). The G protein is inserted as trimers into the viral membrane and it is the only one expressed at the virion surface [117, 118]. The G protein encases the virus and triggers the attachment and entry of the virus into the host target cells by binding to a cellular receptor (receptor-mediated endocytosis), which has been identified as fibronectin for VHSV [118, 119]. The G-mediated fusion process depends on the pH, thus VHSV G protein at pH 6.5 is exposed at the cell surface in an inactive fusion state, and at pH > 7 at the cell surface is needed to shift towards an activated state [120]. The G protein is also the major antigenic protein of novirhabdoviruses, eliciting strong innate and adaptive host immune responses [85, 121] and being the only protein responsible for the synthesis of neutralizing antibodies, which constitute the main humoral component of the fish long-term protective response against rhabdoviruses. Studies with subunit and DNA vaccines have clearly indicated that the G protein alone is necessary and sufficient to stimulate protective immunity for VHSV, IHNV and HIRRV [85, 122-126]. However, vaccination with the recombinant G protein did not protect as well as when using the intact virus, probably because of inappropriate folding as suggested by Boudinot et al. 2004 [127]. Moreover, targeted mutations on G allow attenuation of virulence [128-130].

The matrix (M) protein**,** is localized between the inner leaflet of the surrounding lipid bilayer membrane and the nucleocapsid. M protein normally functions in viral assembly by binding the ribonucleoprotein core of the virus to the host plasma membrane during the budding process

[131]. The M protein interacts with the G protein to assist in viral budding [73]. Apart from its critical role in viral assembly and budding, the M-protein has been also implicated in anti-host functions (or cytopathic functions) that are genetically separable from viral assembly functions (and vice versa) [132] and are still essential for efficient viral replication. Thus, in Vesicular Stomatitis virus (VSV), the prototype rhabdovirus, the M protein appears to block host gene expression [132-134] and/or inhibits export of mRNA from the nucleus [135], effectively blocking translation of host proteins—including those thought to be involved with the viral response pathway [136]. Moreover, VSV M protein induces apoptosis, which probably correlates with the ability to inhibit host gene expression [137]. According to Chiou et al. 2000 [138], the IHNV M protein induced the inhibition of host-directed gene expression and stimulated apoptosis when transfected into a fish cell line. This indicates a role for the IHNV M protein in immune evasion strategies similar to that of the VSV M protein. Meanwhile, several M variants have been found for VHSV [107] but little else was known about VHSV anti-host processes until Pore 2012 [139] and Ke et al. 2017 [140] reported that VHSV M protein acts similarly to other rhabdoviral M proteins (such as VSV, rabies and IHNV) in shutting down general host transcription, the inhibition of interferon promoter and assist in viral budding [73, 139]. In addition, these authors also established that VHSV M protein expressed after transfection located to nucleus and cytoplasm.

4. *Life cycle of VHSV*

Understanding the viral infection cycle is important to define where the host immune system encounters virus-associated molecules. The general rhabdovirus life cycle, derived from VSV, has been reviewed extensively elsewhere and involves the five structural proteins found in all rhabdoviruses [141]. Briefly, the rhabdovirus enters its target cell by receptor-mediated endocytosis, which is triggered following engagement of a cell surface receptor by the viral G protein. The viral and endosomal membranes subsequently fuse and the viral nucleocapsid is released into the cytoplasm of the host cell. Within the cytoplasm, the viral genes are sequentially transcribed from the genome using an RNA-directed RNA polymerase that accompanies the infecting virion, viral proteins are synthesized by host cell machinery, and new copies of the genome are synthesized from a full-length, single-stranded RNA anti-genome. To package the new virions, the N, L and P proteins, synthesized by free ribosomes in the cell cytoplasm, bind to the newly synthesized copies of the viral RNA genome to form the ribonucleoprotein (RNP) core, which associates with the M protein to produce the RNP-M complex. The G protein is synthesized by endoplasmic reticulum (ER)-bound ribosomes. It is glycosylated and further modified within the ER and Golgi apparatus prior to transport and insertion into the plasma membranes on the host cell surface. The RNP-M complex migrates to regions of the plasma membrane enriched with viral G proteins. The G protein-studded host cell plasma membrane is subsequently captured by RNP-M protein complex as it buds from the cell to create fully enveloped rhabdovirus **(Figure 6).**

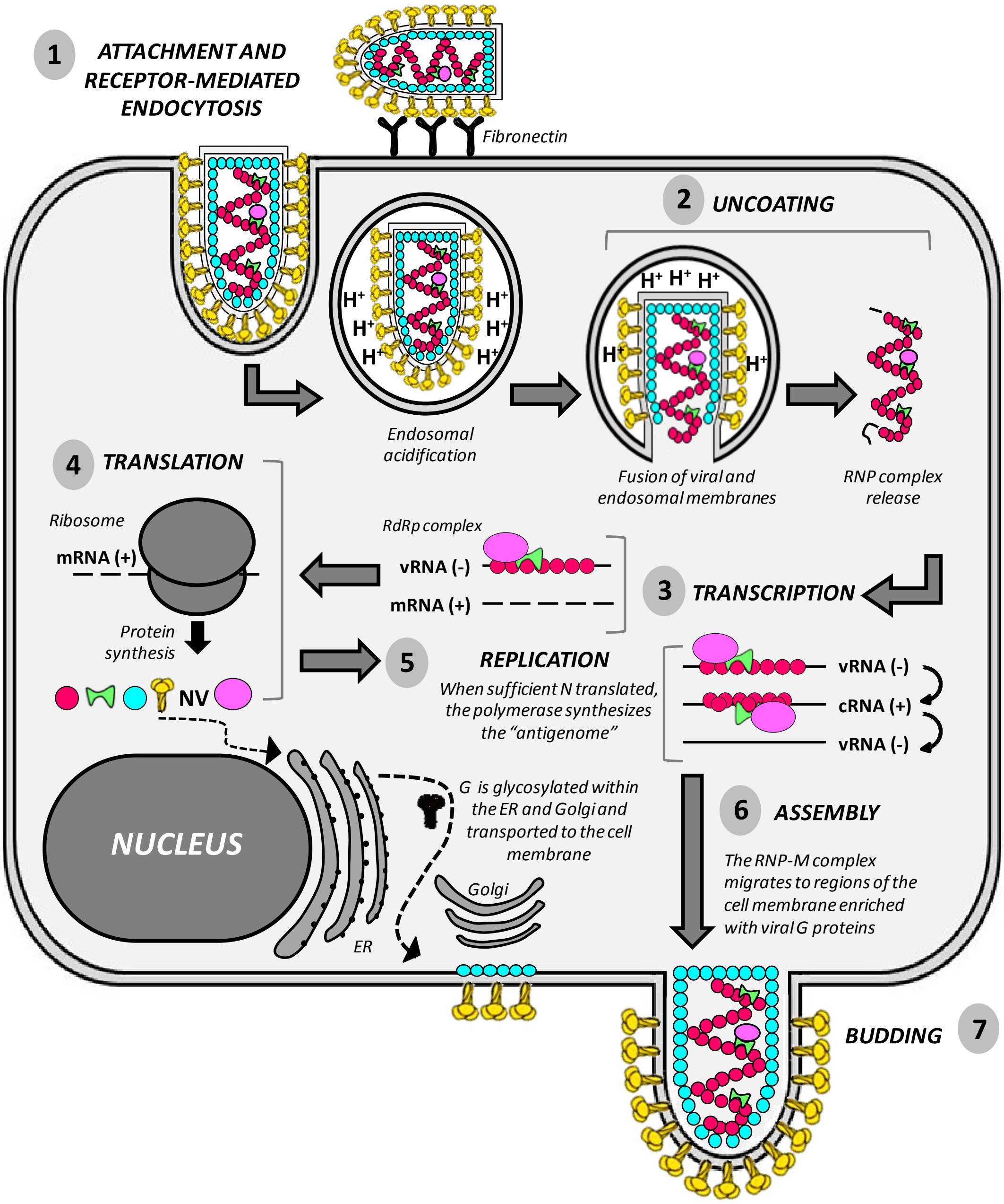

Figure 6. The lyfe cycle of VHSV consist of: (1) attachment and receptor-mediated endocytosis, (2) uncoating, (3) transcription of viral genome, (4) translation of viral proteins, (5) replication of viral genome, (6) virion assembly and, finally, (7) budding. (1) The glycoprotein (G) binds to a cell surface receptor (fibronectin) allowing viral endocytosis into the host cell. **(2)** Endosomal acidification triggers a conformational change of the G that fuses the viral envelope with the endosomal membrane and releases the viral ribonucleoprotein (RNP) complex (consisting of the N, P, and L proteins and the negative-sense vRNA) into the host/cell cytoplasm. **(3)** Within the cytoplasm, the nucleoprotein (N) dissociates from the vRNA (-) and the phosphoprotein (P) activates the viral polymerase (L) to initiates the synthesis of positive-sense transcripts of all six viral genes (mRNA). **(4)** Viral proteins are

synthesized by host cell machinery (ribosomes) from mRNAs. The G protein is synthesized by endoplasmic reticulum (ER)-bound ribosomes, glycosylated and further modified within the ER and Golgi apparatus and finally transported and inserted into the host cell membrane. **(5)** When sufficient N has been translated to allow nascent vRNA (-) encapsidation, L synthesizes a full-length positive, complementary copy called the “antigenome”, cRNA (+). The antigenome is used as a template to direct synthesis of encapsidated minusstrand genome and for the synthesis of both new mRNAs (secondary transcription) and antigenomic RNA. **(6)** To package the new virions, the N, L and P proteins, synthesized by free ribosomes in the cell cytoplasm, bind to the newly synthesized copies of the vRNA (-) to form the ribonucleoprotein (RNP) core/complex, which associates with the matrix protein (M) to produce the RNP-M complex. The RNP-M complex migrates to regions of the plasma membrane enriched with viral G proteins and the M binds the cytosolic domain of G, linking the viral genome and its associated proteins responsible for encapsulating the virion with the host membrane. **(7)** Fully formed and host-membrane encapsulated virions are released to infect new cells.

5. Genotypes and serotypes

There are three recognized serotypes of VHSV defined by means of different versions of the neutralization test: Type 1, represented by Strain F1; Type 2, represented by the Hededam isolate, both isolated from Danish rainbow trout [142, 143], and Type 3, represented by the French strain 23/75 isolated from brown trout *Salmo trutta* far10 by De Kinkelin and Le Berre 1977 [144]. However, in spite of the existence of serological differences between isolates, serological cross-reactivity occurs among the three serotypes and does not allow discrimination between marine and freshwater isolates [145]. Because VHSV isolates from wild marine fish are serologically indistinguishable from the continental isolates [26, 146-148], development of molecular methods based on the analysis of sequences of viral genes provide the most discriminatory way of typing VHSV.

Phylogenetic studies based on sequencing of full-length or truncated genes from the N gene [149-151], G gene [47, 149] and NV gene [149] of VHSV isolates worldwide showed that VHSV can be classified into four major genotypes (designated from I to IV) [47, 150, 152, 153]. Additionally, genotypes I and IV are further divided into five (Ia-Ie) [47] and three (IVa-IVc) [24] sublineages, respectively, based on their reactivity to different monoclonal antibodies [154]. These genotypes are correlated to the geographical regions or areas of isolation rather than host specificity or pathogenicity, with isolates from genotypes I, II and III circulating in Europe and genotype IV consisting of isolates from North America and East Asia [47, 146], as detailed in **Table 3.**

Table 3. Currently known genotypes and distribution of the viral haemorrhagic septicaemia virus (VHSV).

Genotypes	Location	Subtypes	Location	References
I	Europe and Japan (Ib)	Ia	Continental Europe	A
		Ib	Baltic and North Sea and Japan	B
		Ic	Denmark and Germany (some mainland lakes)	C
		Id	Water along Norway and Finland, Baltic Sea.	D
		Ie	Black Sea (Georgia and Turkey)	E
II		---	Baltic Sea and rivers flowing into it	F
III		---	North Sea and NE Atlantic Ocean (restricted to waters connected to the North Atlantic Ocean)	G
IV	North America and East Asia	IVa	West coast of North America (Pacific Ocean) and East Asia (Korea and Japan)	H
		IVb	Great Lakes of North America	I
		IVc	East Coast of North America (Atlantic Ocean)	J

A: [47, 152, 155]; **B:** [47, 51, 152]; **C:** [156]; **D:** [157]; **E:** [158]; **F:** [51, 158, 159]; **G:** [47, 51, 55, 56, 58, 150, 152, 160, 161]; **H:** Pacific Ocean [26, 50, 148, 162] , and East Asia [163, 164]; **I:** [56, 165]; **J:** [24].

Within genotype I, sublineage Ia includes freshwater isolates from continental European countries, and is further divided into two distinct sublineages: Ia1, comprising strains isolated mainly from Danish rainbow trout; and Ia2, representing viruses that predominantly originate in continental Europe. Sublineage Ib circulates in marine fish of the Baltic and North Sea and the English Channel. Sublineage Ic is a smaller group consisting of Danish rainbow trout isolates from the 1980s. VHS outbreaks in Finnish sea reared rainbow trout in the Baltic Sea grouped together in genotype Id [166], together with some old Scandinavian isolates from the 1960s. Sublineage Ie is formed by isolates from Georgia and Turkey. In addition, genotypes II and III appear to be restricted to the Baltic Sea and the North Atlantic Ocean. Finally, genotype IV has been isolated from marine and freshwater fish in North America (subtypes IVa, IVb and IVc) [56] and northeast Asia, including Japan and Korea (only subtype IVa) [167]. Genotypes Ib and IVa have also been reported in marine waters of Japan [24, 47].

The host range and the pathogenicity appear, at least to some extent, to be linked to the genotype. In general, isolates of VHSV from rainbow trout are principally members of genotypes Ia, Ic and Id, and the isolates adapted to marine host species are members of genotypes Ib, Ie and II–IV. Moreover, VHSV marine isolates tested so far typically produce little or no mortality in rainbow trout fry following waterborne challenge [145, 168]. This is consistent with the well supported hypothesis that VHS disease in trout farms is due to VHSV host jumps from marine reservoir fish into cultured rainbow trout, followed by adaptation to higher virulence in trout [47]. Interestingly, molecular analysis provides evidence that the marine environment is the original reservoir of VHSV and that species barrier jumps may have occurred several times, mainly from marine fish to trout [169]. Finally, it is still not clear whether VHSV isolates represent three serotypes or subtypes within a single serotype [170]. Thus, VHSV isolates share several antigenic epitopes, although serogrouping does not correlate with genotypes.

In recent years, genotype characteristics have been used to evaluate viral properties and molecular epidemiology of VHSV from both freshwater and marine fishes. Although genotyping is useful, a quick and simple detection method that can discriminate the different genotypes is desirable for a more efficient prevention of the spread of genotypes to new geographical areas. Some authors [171] demonstrated the possibility of producing MAbs (monoclonal antibodies) that recognize a specific genotype or subtype of VHSV; i.e.: MAb VHS-10, which reacted only with genotype IVa in indirect immunofluorescence (IFI) and ELISA. Using cell cultures that were transfected with each of the viral protein genes, it was shown that the MAb VHS-10 recognizes a nonlinear genotype IVa-specific epitope on the VHSV N protein.

6. Immunity to fish rhabdoviruses: innate and adaptive responses

The protective immune mechanisms against viruses in fish are mounted in similar fashion than those observed in mammals [172]. Teleost fish are known to possess the major TLR families plus an expanded set of unique non-mammalian TLR genes and gene variants [173, 174], as well as pattern recognition receptors (PRRs) that include the cytoplasmic viral detection receptors retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5), laboratory of genetics and physiology 2 (LGP2) and the associated signaling molecule mitochondrial antiviral signaling (MAVS) protein [74, 175, 176], which are commonly referred to together as “RIG-I-like helicases (RLHs)” [177]. RLHs contain helicase domains that are capable of “sensing” viral pathogen-associated molecular patterns (PAMPs), particularly dsRNA and uncapped ssRNA. The interaction causes a conformational change in the RLH exposing a Caspase Activation and Recruitment Domain (CARD) allowing it to signal to the downstream adaptor protein, mitochondrial antiviral signaling (MAVS) protein (also known as VISA/IPS-1/Cardiff) [178].

The cascade activates transcription factors including Interferon-Regulatory Factors (IRFs), resulting in transcriptional activation of responsive genes, type I IFNs in particular [179, 180]. This “first phase” of the innate immune response occurs primarily in infected cells, and many of the above signaling components have been identified in, and/or cloned, in fish as well as humans, mice and other organisms. Overexpression of the mentioned proteins in cultured cells results in a decreased viral titer following infection, suggesting that the antiviral roles they play in mammals originated in earlier vertebrates, such as fishes [74, 181]. Characterization of the innate immune response in fish rhabdoviruses has been accelerated due to the genomic information available for model and commercially important fish species. Genes encoding critical cytokines, chemokines, and other innate effectors have been identified in fish including those encoding both type I IFNs (also called IFNϕ) and type II IFNs (also called IFNγ) [182, 183]. Fish type I IFNs (IFNϕ) are split into two major groups, which differ in the number of conserved cysteine (C) residues (group I—2C and group II—4C) [184] and appear to bind different receptors [185, 186]. In general, group I IFNs are expressed in a wide range of tissue types while group II IFNs are more abundant in hematopoietic-derived tissues and cells. Multiple gene copies of IFNγ exist in fish species but these genes are not duplicated to the same extent as the type I IFNs [184].

Once type I IFNs are transcribed and translated, their protein products are processed and secreted from the cell into the surrounding tissues and to circulation. IFNs bind to high affinity cell surface receptors on neighbouring or distal cells and activate JAK1 and TYK2 tyrosine kinases leading to the phosphorylation of STAT1 and STAT2. STAT1 and STAT2 dimerize and then complex with IRF9 to form the multimeric transcription factor complex IFN stimulated gene factor 3 (ISGF3). After translocation to the nucleus, ISGF3 transcriptionally upregulates hundreds of IFN-stimulated genes (ISGs) [187]. The protein products of these genes work in concert to induce physiological changes that lead to an antiviral state for the cell [188].

The IFNs signal through the conserved JAK/STAT pathway and up-regulate the expression of >300 IFN stimulated genes (ISGs) [189, 190], which establish the ‘anti-viral’ state [191]. Many of these ISGs have direct anti-viral function (i.e.: Mx [192]) while the functions of other ISGs remain unknown. Replication of fish rhabdoviruses is inhibited by pre-activation of the IFN system regardless of the method used, including using IFN-containing supernatants, poly I:C [193], rhabdoviral G protein transfection [194-196] or recombinant IFN [197, 198]. Early studies clearly indicate functional IFN in sera derived from fish infected with VHSV [199], IHNV [200] or SVCV [201]. Later studies confirmed these results and demonstrate transcriptional up-regulation of both group I and II IFN genes after infection with these same viruses [202-204]. IFNγ genes are also regulated early in rhabdoviral infection [203]. Rhabdoviral infections are controlled by fish group I and II IFNs [197, 202, 204-206]. Host-virus dynamics at virus entry points (i.e., the fins [40]) may help limit the internal spread of viral infection by alerting systemic sites via IFNs and other cytokines.

Other important aspect in long-term immunity against rhabdoviruses is the humoral immune response. Neutralizing antibodies induced by infection and/or vaccination are critical components of long-term adaptive immunity to fish rhabdoviruses [207]. Passive immunization with sera containing neutralizing antibodies protects recipient juvenile trout from IHN and Pacific herring from VHS, even when titers fall below detectable levels [208, 209]. Most studies of fish rhabdoviruses have focused on the highly protective neutralizing antibody response but it is possible that non-neutralizing or other types of antibodies may also play a role. *In vitro* rhabdoviral neutralization is complement-dependent but the exact mechanism by which complement aids viral neutralization is still unclear [210, 211]. Neutralizing antibodies are unlikely to play a role in surviving the acute infection phase in coldwater fish species since neutralizing antibodies typically are not detectable until several weeks post-infection.

The study of attenuated or low virulence virus types indicates that a certain threshold of virus replication must occur for fish to develop a broadly protective antibody response, even if a robust innate response is induced [212]. Lorenzen and LaPatra published a comprehensive review of the antibody response to fish rhabdoviruses in 1999 [211], and since then, this area has not received much new attention. Studies of the G protein DNA vaccines over the past decade have reaffirmed the central role of neutralizing antibodies in protective immunity [85, 213-217]. However, genomic approaches combined with functional studies have brought exciting new insights into B cell biology in teleost fish. Fish B cells show phagocytic behaviour suggesting that fish B cells may also function as part of the innate immune system [218]. Furthermore, it is now known that teleost fish possess three or more immunoglobulin isotypes including IgM, IgD and IgT (IgT is also called IgZ in zebrafish) [219]. A recent study indicates that B cells expressing IgM respond to antigenic stimulation in systemic tissues while B cells expressing IgT are key to the mucosal immune response [220]. To date, tools such as monoclonal antibodies to fish IgM and IgT now exist [220, 221], which will allow the characterization of mucosal immunity and its relationship to systemic protection against rhabdoviruses. Finally, there has been much progress in unraveling the complexity of the fish complement system [222], which may help to finally define which complement components contribute to virus neutralization.

The cellular immune response to fish rhabdoviruses also has a key role in protection. Although DNA vaccination with the novirhabdoviral G protein triggers production of protective neutralizing (and perhaps other) antibodies, high levels of specific protection after DNA vaccination are also observed without detectable neutralizing antibodies [78, 223] indicating a potential role for specific cellular immunity. Major advances have been made recently in our understanding of fish T cells [224]. Teleost fish possess a wide range of T cell associated genes, including genes that encode T cell receptor chains and various other T cell associated co-receptors, co-stimulatory molecules and cytokines [224]. Many of these genes show up-regulated expression following rhabdoviral infection and/or G protein DNA vaccination [225]. There is a limited understanding of the role T cells play during fish rhabdoviral infection with most work focusing on cell-mediated cytotoxicity (CMC). However, VHSV infection and DNA vaccination with the VHSV G protein induces clonal expansion of T cells, as shown by spectratype analysis of the complementarity-determining region 3 (CDR3) of the TCR-β chain [226, 227], supporting a role of teleost T cells in controlling this virus. Interestingly, the dominant CDR3 profiles are the same for both VHSV infection and G protein DNA vaccination suggesting that important T cell epitopes are localized within the G protein. Future studies examining the role of T cells during rhabdoviral infection will benefit from new reagents that can discriminate among T cell subsets [224], including monoclonal antibodies against the CTL surface marker CD8.

Studies of mammals and other vertebrate species indicate that NK cell activity is mediated by many different receptor types that can be specific to an individual animal species or even to a cell lineage [228]. Candidate NK receptor families in fish include the novel immune type receptors (NITRs), novel Ig-like transcripts (NILTs), leukocyte immune type receptors (LITRs), and possibly others [228]. Genomic characterization of fish species has enabled the identification of these polymorphic and polygenic receptor families, but there has been limited validation that these receptors have a functional role in NK activity. Functional studies of specific CMC were made possible in fish following the development of isogenic fish and major histocompatibility complex (MHC) I matched cell lines for both crucian carp and rainbow trout [229-231]. In a previous study, peripheral blood lymphocytes from rainbow trout infected with VHSV exhibit significant CMC to MHC class I matched target cells infected with VHSV but not to VHSV infected xenogeneic targets [231]. However, rainbow trout survivors re-exposed to VHSV mounted a kinetically faster, VHSV-specific CMC response, as well as a nonspecific CMC response against VHSV infected xenogeneic targets (starting at 11 days post-infection). The delayed nonspecific CMC response in trout contradicts the mammalian paradigm where primary CMC is nonspecific and results from NK cell activity while later CMC is pathogen-specific due to clonal expansion and action of virus specific CTLs [232]. Taken together, these studies suggest a role for cellular immunity—particularly CMC—in the host response to fish rhabdoviruses. However, more basic research is needed to definitely identify the cells that contribute to key cellular effector functions in fish.

C. THE NV GENE OF NOVIRHABDOVIRUS

1. NV gene and protein

The non-virion (NV) gene is approximately 500 nucleotides in length and codes for a small protein of 111 amino acids (13.2 kDa) for IHNV, 122 amino acids (13.6 kDa) for VHSV, 120 amino acids (13.7 kDa) for HIRRV, and 122 amino acids (13.6 kDa) for SHRV [95, 97, 233]. The NV protein is not present in the virion particles, as its name suggests [46, 96, 97, 234]. Despite of the presence of the NV gene in the four *novirhabdovirus* species mentioned above as a common characteristic, NV proteins showed relatively high homology intraspecies but widely divergent interspecies sequences [149, 233].

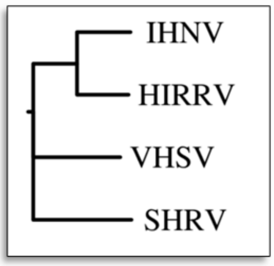

Figure 7. Phylogenetic tree depicting the homology and clustering among four NV amino acid sequences of novirhabdovirus: VHSV NV (07.71 strain, GenBank AJ233396), HIRRV NV (GenBank U47847.1), IHNV NV (Oregon69 strain, Uni-ProtKB Q08455), and SHRV NV (GenBank NC_000903.1).

NV gene was firstly described from IHNV by Kurath et al. 1995 [95] and further completely sequenced in the same year [235, 236], being one year later identified for VHSV NV gene [97]. Regarding homology among NV proteins, initial studies pointed out less than 20% amino acid identity according to Morzunov et al. 1995 [236], and more complete identity studies defined values between 15% and 50% for VHSV vs IHNV (16.3 %), IHNV vs HIRRV (51.3 %), VHSV vs HIRRV (15.7 %) and VHSV vs SHRV (23,2 %) [233] **(Figure 7)**. In spite of this, NV from different novirhabdovirus (except for SHRV) seems to be implicated in pathogenesis [237, 238] leading to similar effects as discussed below. In Europe and USA, VHSV and IHNV have caused important outbreaks in the last decades, which have allowed their study and the characterization of multiple NV sequences. To date, there are more than 60 and 120 amino acid accession entries for IHNV NV (https://www.ncbi.nlm.nih.gov/protein/?term=NV+IHNV) and VHSV NV (https://www.ncbi.nlm.nih.gov/protein/?term=NV+VHSV), respectively.

2. NV role: replication, pathogenicity and immune response

The presence of a functional NV gene with the same location and similar size in all novirhabdovirus, which have relatively small genomes where over 90% of the nucleotides are protein-coding, suggested that the conserved NV gene in this genus could play a critical function for novirhabdovirus [239]. However, several observations were contradictory for a biological role of NV, such as the absence of amino acid sequence homology among NV proteins from different novirhabdovirus and the lack of a gene encoding NV in some fish rhabdovirus like the spring viremia of carp virus (SVCV). For these reasons, several investigations have been done to uncover the role of this protein.

One of the first evidences of localization and expression of NV from both IHNV and VHSV was carried out by Schutze et al. 1996 [97], since they could detect specifically those NV proteins in infected RTG-2 and EPC cells with VHSV and IHNV, respectively. In that report, the authors observed a nuclear and cytoplasmic expression pattern for IHNV NV, and limited to cytoplasm for VHSV NV in the absence of cross-reaction. Studies carried out many years later confirmed those expression patterns and established their correlation with function for IHNV [238].

A first approach to demonstrate a biological role of NV was carried out by Chiou et al. 2000 [138], in whose work fish cells transiently transfected with a plasmid expressing the IHNV NV gene were found to undergo cell rounding. That suggested a possible interaction between NV protein and the cytoskeleton, and explained the differences found in the cytopathic effect (CPE) induced by recombinant IHNV (rIHNV) viruses expressing NV_{IHNV} or NV_{VHSV}, being the aspect of the rounding cells more pronounced in the presence of the NV of VHSV [240].

However, it has not been possible to delve into the NV function until a reverse genetics system was established for the recovery of a non-segmented negative-stranded RNA virus: the rabies virus [241]. Since then, this reverse genetics system has been adapted to numerous fish RNA viruses [242] such as the novirhabdovirus SHRV [239], IHNV and VHSV [75, 243-245]. The ability to recover live virus from a DNA copy of the RNA genome (called "reverse genetics"), opens up the possibility of deleting genes to study their function among other applications.

The NV role has been then examined using deletion mutants of different novirhabdovirus generated by this technology mentioned above [242], but their results differed substantially among viruses (e. g.: SHRV vs VHSV). While NV was not essential for *in vitro* or *in vivo* SHRV production on warm-water flatfish [239, 246], the recovery of IHNV and VHSV NV-deletion mutants demonstrated that NV protein was dispensable but necessary for an efficient virus replication in cell culture [237, 240, 244, 245, 247] and essential for virus pathogenicity in rainbow trout [40, 240], yellow perch [237] and Japanese olive flounder [247] **(Table 4)**. Thus, recombinant IHNV and VHSV lacking whole NV gene showed severely retarded growth (one to two logs lower in cell culture than the wild-type counterparts) compared to WT and exhibited highly attenuated phenotypes in fish, which were also shown to have a limited persistence in internal organs of the fish [42, 247] or even to be blocked in the fins without any further spread into the infected fish [40]. However, the role of HIRRV NV in replication and pathogenicity has not been studied yet.

Table 4. Summary of all the studies in which NV gene was knocked-out and the recombinant viruses recovered by reverse genetics to study the role played by the NV protein on novirhabdovirus replication *in vitro* and on pathogenicity *in vivo*.

REFERENCES	Virus	Recombinant Viruses	NV ROLE		
			in vitro	*in vivo*	Conclusion
Johnson et al. 2000 [239]	SHRV	rSHRV-truncated NV ORF	Replicated in EPC cells	-----	**Not required for efficient viral replication and pathogenesis**
Alonso et al. 2004 [246]		rSHRV- ΔNV		Virulent in zebrafish	
Biacchesi et al. 2000 [244]	IHNV	rIHNV-ΔNV rIHNV-ΔNV-GFP rIHNV-ΔNV-CAT	Replicated poorly in EPC cells	-----	**Required for efficient viral replication and pathogenesis**
Thoulouze et al. 2004 [240]		rIHNV-ΔNV-GFP		Highly attenuated in rainbow trout	
Choi et al. 2011 [238]		rIHNV-NV-ΔEGDL rIHNV-ΔNV-GFP	Replicated poorly in RTG-2 cells	-----	
Wu et al. 2018 [248]		rIHNV-NV-ΔEGDL	Stable replication in CHSE-214 cells	Attenuated in rainbow trout	
Biacchesi et al. 2010 [245]	VHSV	rVHSV-ΔNV	Replicated poorly in EPC cells	-----	
Ammayappan et al. 2011 [243]		rVHSV-ΔNV-EGFP		Highly attenuated in yellow perch	
Kim et al. 2011 [247]				Highly attenuated in olive flounder	

Although these ΔNV-viruses were highly attenuated, they were all able to efficiently express detectable amounts of foreign proteins instead of the NV in cell cultures [245] and to induce neutralizing antibodies when injected into fish, allowing their use as vaccine vectors ([42, 244, 249-251] and Harmache and Brémont, unpublished data). In fact, these NV-knockout variants have then been used to immunize fish against IHNV and VHSV, and a high degree of protection has been obtained in Japanese flounder against VHSV [42] and rainbow trout against IHNV [240].

However, even though the NV deletion constitutes an attractive approach for the generation of safe live vaccines by attenuating virus virulence irreversibly while retaining protective properties, this process could unfortunately reduce its immunogenicity due to the reduction of viral antigens, as observed for rIHNV-ΔNV *in vitro* [240].

Interestingly, the IHNV and VHSV NV proteins (and no SHRV NV) share a similar function despite of the low percentage of homology between them (23%) [149, 233, 240, 242], since a recombinant IHNV virus in which the IHNV NV gene was replaced by that of VHSV (rIHNV-NV_{VHSV}), was shown to replicate efficiently in fish cells and to induce similar cumulative percentage of mortality *in vivo* in rainbow trout compared to the wild-type (WT) IHNV [240].

However, the interference with replication *in vitro* and pathogenicity *in vivo* are not the only known and proven effects of NV proteins. Comparative studies between wt and NV knockouts of IHNV and VHSV suggest that NV proteins may downregulate the induction of interferon (IFN) and interferon-stimulated genes (ISGs) *in vitro* and *in vivo* [238, 251], which are considered the first line of host defense against viral infection. Teleost fish IFNs can induce the expressions of several ISGs with antiviral activity. Among ISGs, those coding Mx proteins play an important role due to their direct antiviral activity and they have been considered as markers of IFN I activity in some fish studies [252, 253]. Thus, Choi et al. 2011 [238] observed that NV knockout IHNV induced higher levels of expressions of both *ifn1* and *mx1* genes than wild-type during *in vitro* infection of rainbow trout gonad cells (RTG-2). Similarly, Kim and Kim 2012 [251] observed the up-regulation of interferons and *mx* transcript levels during *in vitro* infection of cyprinid cells (EPC) and *in vivo* infection of olive flounder with NV knockout VHSV. Furthermore, according to Choi et al. 2011 [238], the inhibition of virus-induced IFN response depends on the NV nuclear localization in the infected cell, being the ^{32}EGDL^{35} motif within NV responsible for driving IHNV NV to the nucleus. Although VHSV NV was reported to restore the normal growth of NV knockout IHNV [240] probably via inhibition of host IFN system as IHNV does (as it has been hypothesized by Choi et al. 2011 [238]), the underlying mechanisms of IFN inhibition of VHSV NV may be different from those of IHNV NV.

It is unclear whether other novirhabdoviruses (apart from IHNV and VHSV) use the NV to inhibit the IFN system, but in the case of SHRV, an NV-knockout virus showed the same pathogenicity as wild-type virus, suggesting that this virus may cope with the host antiviral response using proteins other than NV [246]. But, it should be noted that the pathogenicity of the SHRV-ΔNV was assayed on zebrafish and by intraperitoneal injection, which do not reflect the SHRV natural host (snakehead fish) and its presumed route of infection (waterborne) [246].

Other recent evidences also propose that VHSV NV protein suppresses TNFα-mediated NF-κB activation [254]. These authors showed that pre-treatment of EPC cells with TNFα prevented CPE caused by VHSV infection, but no protective effect was observed when cells were treated with TNFα after VHSV infection, suggesting that immediate early TNFα-mediated responses inhibit VHSV replication and thus VHSV should possess an ability to evade or inhibit early TNFα-mediated antiviral responses to promote a successful replication in host cells. Those results were different from the results reported by Roca et al. 2008 [255] in which TNFα treatment resulted in increased susceptibility of zebrafish to SVCV infection and increased viral replication in ZF4 cells. However, even though SVCV is a member of the family *Rhabdoviridae*, it belongs to *Vesiculovirus* genus whose genome does not possess the NV gene [106, 256]. Therefore, there might be differences between VHSV and SVCV in the exploitation of TNFα-mediated signaling pathways. Furthermore, Kim and Kim [254] observed that the NV knockout VHSV (rVHSV-ΔNV-EGFP) induced significantly higher NF-kB activity in EPC cells than the wild-type. This would suggest that VHSV adopts a strategy to suppress early activation of NF-kB in host cells through NV protein.

Recently, Ammayappan and Vakharia (2011) [237] established that the NV protein had an anti-apoptotic function at early stages of infection in vitro. In that work, the authors demonstrated that NV knockout and NV-deficient viruses cause greater apoptotic effects than wild-type virus (rVHSV), as shown by DNA laddering and downregulation of caspase 3, 8, and 9 activities. Moreover, a recombinant VHSV expressing the IHNV NV protein (chimeric rVHSV-NV IHNV) exhibited similar properties (growth, caspase induction, DNA fragmentation and viral protein synthesis) to those of the wild-type rVHSV, suggesting that NV protein of novirhabdovirus is capable of suppressing apoptosis non-specifically in EPC cells.

By performing transfection experiments using an expression vector that codes for the IHNV NV protein, it was also observed that the expression of NV in trans can completely restore the antiapoptotic activity of VHSV during infection (data not shown by Ammayappan and Vakharia 2011 [237]. Similarly, Romero et al. 2008 [250] observed that EPC cells infected with wild-type IHNV produced lower levels of apoptosis than recombinant IHNV carrying the green fluorescence protein (GFP) gene rather than the NV gene [250]. Moreover, the early apoptosis observed by Ammayappan and Vakharia (2011) [237] was accompanied by decreased production of viral progeny. These findings suggest that the antiapoptotic function of the NV protein observed *in vitro* can also correlate with the attenuation of the virus observed *in vivo* by Amappayapan et al. 2010 [75] and that the NV protein plays an indirect role in the pathogenesis of novirhabdovirus by promoting the viral growth by delaying the cellular apoptosis program.

3. Molecular mechanism of NV proteins

NV proteins (except SHRV NV) have previously been shown to be involved in viral pathogenicity [40, 237, 240, 244, 245, 247], but the mechanisms by which NV proteins interfere with the innate host responses and, therefore, promote viral replication remain still unclear. Our study published in 2015 [257] and that is part of the present Thesis, aimed to find out in transcriptome possible targets implied in the inhibition of immune response in trout. Biacchesi et al. 2017 [258] developed an approach based on reverse genetics and interactomics and identified several NV-associated cellular partners as potential targets. Among these cell partners, they showed that NV proteins specifically interact with a protein phosphatase, Mg^{2+}/Mn^{2+}-dependent, 1Bb (PPM1Bb, PPC2 family) and recruit it in the close vicinity of mitochondria, a subcellular compartment important for retinoic acid-inducible gene-I- (RIG-I)-mediated interferon induction pathway. PPM1Bb protein have recently been shown to negatively regulate the host antiviral response via dephosphorylating Traf family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) [259, 260]. They showed how VHSV and IHNV NV proteins seem to interact with PPM1Bb in a different manner; VHSV NV recruits PPM1Bb around mitochondria to dephosphorylate TBK1, and further interferes with the retinoic acid inducible gene-I (RIG-I) response, whereas IHNV NV could bind to PPM1Bb around mitochondria and nucleus, acting on nuclear substrates different from TBK1 for inhibiting RIG-I response [258]. In spite of these findings, more knowledge is needed about other molecules implied downstream TBK1 as mediators in signaling pathways, helping to define possible relationships between them.

II. OBJECTIVES AND JUSTIFICATION

Novirhabdovirus are fish pathogens of great importance for aquaculture worldwide. Previous studies defined NV proteins (except SHRV NV) as responsible for *in vivo* pathogenicity of novirhabdovirus and also showed a clear relationship between their subcellular location and function. Despite the low homology shared between NV proteins, they conserve the same function. For all the reasons mentioned above, this thesis work aims to achieve the identification of the VHSV NV regions and mechanisms involved in the early shut-off of the host antiviral response carried out by the NV protein of VHSV.

The main objectives of this thesis work are:

1.	To express and characterize VHSV NV (07.71) protein obtained in prokaryotic systems.
2.	To characterize the whole immune-related transcriptome profile induced by NV (07.71) in trout by using an immune-enriched microarray.
3.	To define the relationship among immune genes and pathways modulated in response to NV protein in trout.
4.	To correlate the VHSV NV (07.71) protein sequence with its function by using different fragments and to determine the effects of fragments on transcription of immune response genes.
5.	To study the functional implications of VHSV NV (07.71) variants compared to other novirhabdoviruses.
6.	To correlate the subcellular location of NV fragments and variants with function. To establish the differences with those NV proteins from other novirhabdoviruses.
7.	To develop a method for the detection of VHSV neutralizing antibodies in low amounts of blood and large number of fish samples.

III. SUMMARY OF PAPERS:

METHODOLOGY AND RESULTS

PAPER I: *Transcriptomic study of VHSV NV in trout*

TRANSCRIPTOME ANALYSIS OF RAINBOW TROUT IN RESPONSE TO NON-VIRION (NV) PROTEIN OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV)

El virus de la hemorragia septicémica viral (VHSV) infecta un gran número de especies acuáticas, siendo la trucha arcoíris uno de los salmónidos más afectados en cuanto a mortalidad. La proteína no-viriónica (NV) de VHSV no es esencial para la replicación del virus *in vitro*, pero sí lo es para la patogenicidad *in vivo* de VHSV. El objetivo de este trabajo es caracterizar en trucha los efectos que induce la NV en el transcriptoma de genes inmunes y de otros genes relacionados. Para el desarrollo del trabajo disponíamos de un microarray de trucha propio, diseñado y validado en nuestro laboratorio, capaz de reconocer un mayor número de genes inmunes que los microarrays comerciales. Inicialmente, se obtuvo la proteína NV de VHSV (07.71) para realizar los experimentos en trucha mediante inyección intraperitoneal. Se recogieron el bazo y el riñón cefálico de las truchas tratadas con NV y de las truchas control. Se extrajo el RNA total y sólo aquellas muestras que cumplían con los criterios de calidad se hibridaron con el microarray. El análisis estadístico de los datos obtenidos a partir del microarray permitió establecer los resultados mostrados en esta publicación, siendo estos novedosos y no habiendo sido descritos con anterioridad en la literatura. La proteína NV indujo una regulación negativa de la mayoría de los genes del microarray, si bien algunos se encontraban regulados positivamente. Entre los nuevos genes downregulados se encontraban genes clasificados como: VIG, factores de necrosis tumoral, receptores tipo-Toll, genes de procesamiento y presentación antigénica, interleuquinas, citoquinas, factores de transcripción, etc. Algunos de estos genes (*stat1, jun y atf1*) participaban en múltiples vías de activación (genes multipath), siendo regulados negativamente por la acción de la NV. En conjunto, todos estos resultados contribuyen a definir la función y efectos de la NV en trucha. De este modo, la extensa downregulación de genes inmunes inducida por la NV conllevaría la alteración del normal desarrollo de las respuestas inmunes innata y adaptativa, para así favorecer la replicación de VHSV. Además, este estudio pone de manifiesto el gran potencial de las metodologías “big data” para esclarecer, entre otros, los mecanismos moleculares implicados en la respuesta inmune a rhabdovirus.

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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis of rainbow trout in response to non-virion (NV) protein of viral haemorrhagic septicaemia virus (VHSV)

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Abstract The non-virion (NV) protein of viral haemorrhagic septicaemia virus (VHSV), an economically important fish novirhabdovirus, has been implicated in the interference of some host innate mechanisms (i.e. apoptosis) in vitro. This work aimed to characterise the immune-related transcriptome changes in rainbow trout induced by NV protein that have not yet been established in vivo. For that purpose, immune-targeted microarrays were used to analyse the transcriptomes from head kidney and spleen of rainbow trout (*Oncorhynchus mykiss*) after injection of recombinant NV (rNV). Results showed the extensive downregulation (and in some cases upregulation) of many innate and adaptive immune response genes not related previously to VHSV infection. The newly identified genes belonged to VHSV-induced genes (*vigs*), tumour necrosis factors, Toll-like receptors, antigen processing and presentation, immune co-stimulatory molecules, interleukins, macrophage chemotaxis, transcription factors, etc. Classification of differentially downregulated genes into rainbow trout immune pathways identified *stat1* and *jun/atf1* transcription factor genes as the most representative of the multipath gene targets of rNV. Altogether, these results contribute to define the role and effects of NV in trout by orchestrating an immunosuppression of the innate immune responses for favouring viral replication upon VHSV infection. Finally, these transcriptome results open up the possibility to find out new strategies against VHSV and better understand the interrelationships between some immune pathways in trout.

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Keywords Novirhabdoviruses · VHSV · NV · Transcriptome · Rainbow trout · Targeted-microarrays · *stat1* · *jun* · *atf1* · Interferon · *mx* · Caspases · TLR · VHSV-induced genes (VIG) · Interleukins · Chemokines · Complement · Macrophage · *tnf* · Antigen presentation machinery

Introduction

Viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), *Snakehead rhabdovirus* (SHRV) and *Hirame rhabdovirus* (HIRV) are species of a group of enveloped negative-stranded RNA rhabdoviruses affecting fish. They all have a single RNA genome of ~11 Kb (Nishizawa et al. 2002; Schütze et al. 1995, 1999) encoding five virion proteins (N, P, M, G and L proteins) and a non-virion (NV) protein. The presence of the NV gene was the common characteristic that grouped these viruses into the *Novirhabdovirus* genus (ICTV 2009), in contrast to other fish rhabdoviruses which lack NV, such as spring viraemia carp virus (SVCV). Novirhabdoviruses importantly affect wild-type and farmed fish. For instance, VHSV was isolated from more than ~50 fish species from North America, Asia and Europe, including ~15 farmed (Skall et al. 2005) and free-living marine fish species (Brudeseth and Evensen 2002). Some recent outbreaks happened in Asia, and Northern and Eastern Europe. Olive flounder was affected in Japan (Isshik et al. 2001) and Korea (Kim et al. 2009) where the latest outbreaks reported were in 1991 and 2005, respectively. For rainbow trout, some farms had outbreaks in Norway (2007) (Dale et al. 2009), Finland (2004) (Raja-Halli et al. 2006) and Slovenia (2007), where VHSV was eradicated since 1997 (Toplak et al. 2010). Finally, a North American novel strain has been isolated more recently (Pierce and Stepien 2012). VHSV is a notifiable disease to the Office International des Epizooties (OIE, Paris, France). The presence of VHSV in a fish farm leads to the sacrifice of all fish. However, it is difficult to add an economic estimation which really reflects this situation (Wahli and Bergmann 2011).

The NV gene is ~500 nucleotides in length, corresponding to 12- or 14-kDa proteins in IHNV or VHSV, respectively (Kurath et al. 1997). Despite of the NV gene presence in the four rhabdovirus species mentioned above, their NV proteins showed high homology intraspecies but very divergent interspecies sequences (Einer-Jensen et al. 2005; Kurath et al. 1997). Regarding their functionality, while NV was not essential for in vitro or in vivo SHRV production on warm-water flatfish (Alonso et al. 2004), it was required for highest efficient replication of IHNV (Biacchesi et al. 2010; Thoulouze et al. 2004) or VHSV (Kim and Kim 2012) on cold water rainbow trout or olive flounder, respectively. Comparative studies between wild-type (wt) and NV knockout IHNV or VHSV suggest that the NV protein may downregulate host *ifn1/mx* transcriptional levels during in vitro infection of rainbow trout gonad cells (RTG-2) (Choi et al. 2011) or cyprinid cells (EPC) (Kim and Kim 2012), respectively. Furthermore, interferons (IFN)-induced *mx* transcript levels were higher in NV knockout VHSV than in wt-VHSV-injected flounder, suggesting that NV interferes also in vivo with IFN defences to favour VHSV replication (Kim and Kim 2012). It has been also proposed that VHSV suppresses TNF-α-mediated NF-κB activation through NV in vitro (Kim and Kim 2013) and that NV plays an anti-apoptotic function as detected by downregulation of caspase 3, 8 and 9 activities (Ammayappan and Vakharia 2011).

Rainbow trout (*Oncorhynchus mykiss*) is one of the most economically important affected species by novirhabdoviruses such as VHSV. On the other hand, recent data from rainbow trout genomic/transcriptomic studies have increased the number of immune-related messenger RNA (mRNA) sequences available, making it possible the design of immune-targeted microarrays. Classification of the microarray probes into their immune pathways allowed for additional analysis of those genes common to several pathways (multipath genes), as used to study immune responses of zebrafish (*Danio rerio*) to infection with SVCV (Encinas et al. 2013). Because NV may be implicated in modulating more immune-related responses than those mentioned above, we carried out this study to identify the NV-driven transcriptional changes in rainbow trout, by using immune-targeted microarrays (Ballesteros et al. 2012). Since most studies up to date have compared wt- and NV knockout rhabdoviruses despite their different replication rates (~10^5 lower viral titers were obtained in vitro with NV knockout VHSV) (Biacchesi et al. 2010), a novel approach by using recombinant NV (rNV) have been employed here.

In trout, we found that rNV could play to favour viral replication through the downregulation (and upregulation) of a wide variety of innate immune genes and other regulatory genes not described before such as Toll-like receptors (TLRs), interferons, chemokines, interleukins, antigen presentation molecules, macrophage-related, and transcription factors. The expression of some adaptive immune genes is also downregulated. Multipathway gene analysis of downregulated genes suggests that *stat1* and *jun/atf1* genes encode for transcription factors (TFs) that are involved in more signalling pathways and could be inducing the observed wide effects. This work contributes to identify the role of NV in orchestrating a high variety of host downregulation of genes (some upregulated), coding for intracellular, intercellular and humoral proteins with innate and adaptive immune-related functions.

Materials and methods

Preparation of VHSV NV recombinant protein (rNV)

The DNA sequence corresponding to the NV protein of VHSV-07.71 strain isolated from rainbow trout (LeBerre et al. 1977) was used to obtain recombinant NV (rNV, GenBank accession number n° AJ233396). The corresponding NV sequence flanked by *Bam*HI and *Xho*I restriction enzyme sequences was synthesised (Bio S&T Inc., Montreal, Canada) and subcloned into the pRSETa plasmid (Invitrogen™, Life Technologies, Madrid, Spain) that contains a poly histidine tail (polyH) at the 5′-end (N-terminal) for protein purification. The pRSETa-NV construct confirmed by sequencing was used to transform *Escherichia coli* DH5α (Invitrogen™, Life Technologies, Madrid, Spain) by electroporation. High purity plasmid was prepared from *E. coli* pellets by using a modification of a commercial DNA purification system (Promega, Madison, USA). The DNA concentration was then estimated by Nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc., Wilmington, DE, USA).

For expression of the rNV in bacteria, *E. coli* BL21 DE3 cells (Invitrogen™, Life Technologies, Madrid, Spain) were transformed with the pRSETa-NV plasmid, and the protein was expressed and purified following procedures previously described (Rocha et al. 2002). Western blot analysis of soluble and precipitated rNV was performed with anti-6× histidine monoclonal antibody (MAb) (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) and developed by enhanced chemiluminescence (ECL) as detailed before (Encinas et al. 2011). Numerous unsuccessful attempts were carried out to remove the amino terminal polyH tail of 33 amino acids (4 kDa, MRGSHHHHHHGMASMTGGQQMGRDLYDDDKDRW), included digestions with different proteases, solid-phase digestion methods and use of other cloning vectors and/or purification procedures (i.e. Profinity eXact™ pPAL vector from Bio-Rad, Madrid, Spain). The so-called polyH tail was chemically synthesised and used as control to compare with rNV.

Purified rNV did not contain residual LPS (below 0.1 EU/ml) as determined by the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA).

Ethics statement

All procedures used in animals were approved by the ethical and biosecurity committee from INIA (authorisation CEEA 2011/022) and were performed following the National and European Commission guidelines and regulations on laboratory animals' care. All efforts were made to minimise animal suffering. Fingerling rainbow trout were anaesthetised by immersion in 50 mg/ml tricaine methanesulfonate (MS-222, Sigma, Madrid, Spain) and sacrificed by decapitation.

Intracellular incorporation of rNV by rainbow trout head kidney cells

To estimate intracellular incorporation of soluble rNV by rainbow trout head kidney cells, both in vivo and in vitro assays were performed. Thus, while for the in vivo assays, trout were injected with rNV (1 μg of rNV/g of trout) and head kidney cells extracted to be analysed by flow cytometry, for the in vitro assays head kidney cells were first extracted and then incubated with rNV (1–50 ng of rNV per well) before flow cytometry. Cell sorting from head kidney intended to differentiate lymphocytes, macrophages and erythrocytes and if any of these populations acquired the rNV protein. Thus, head kidneys were removed from rainbow trout and their cells isolated by passing the tissue several times through 27G and 30G needles. The cellular suspensions were prepared for in vitro culture by washing with cell culture medium. After being counted with an haemocytometer, the cells were plated (200,000 round cells per well) in a final volume of 100 μl in poly-D-Lys 96-well plates (Corning, NY, USA) and rNV at 5 to 50 ng per 100-μl concentrations added. Plates were incubated overnight at 14 °C in a 5 % CO_2 atmosphere.

In both in vivo and in vitro assays, to stain for intracellular rNV, the cells were first fixed to the solid-phase of the plates by following our published procedure (Chinchilla et al. 2013). For detecting rNV, an anti-6× histidine MAb (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) was added to the wells 500-fold diluted in 2 % FCS, 0.05 % saponin and 0.01 % N_3Na in PBS (permeabilising buffer) and incubated for 60 min. After washing with the permeabilising buffer and manually flicking the plate, rabbit FITC-labelled anti-mouse IgG (Nordic MUbio, Susteren, The Netherlands) was added and incubated for 30 min. After washing with non-permeabilising buffer (same as before but with no saponin), the monolayers were incubated 10 min with 100 μl of 0.25 % trypsin 0.02 % EDTA (Sigma-Aldrich Quimica SA, Madrid, Spain) with strong agitation to suspend the cells for flow cytometry. Trypsin digestion was stopped by the addition of 50 μl per well of 1 % BSA, 50 mM EDTA, 0.01 % N_3Na in PBS and well contents pipetted up and down several times until most cells were suspended. The suspended cells were then analysed in a BD FACSCanto II apparatus (Beckton Dickinson, Madrid, Spain) provided with a high throughput sampler (HTS). Forward (FSC) and side (SSC) scatter threshold values corresponding to damaged cells, cellular debris and/or cellular aggregates (<10 % of total events) were eliminated from the analysis. Cells were gated for FSC/SSC populations (P1–P5) and the number of fluorescent cells in each population over a threshold containing 95 % (mean+2 standard deviations) of non-rNV-treated cells was then determined (Chinchilla et al. 2013; Ruiz et al. 2008). After the multiwell autosampler gated 10,000 events per well of the 96-well plates (60 min), the automatic batch analysis feature of BD FACSDiva calculated the percentage of fluorescent (rNV-positive) cells by the formula 100×number of cells in each population with fluorescence values above the fluorescence threshold/total number of cells gated per population per well. The software exported results to an MS Excel sheet for additional calculations, and those were plotted in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA). Comparisons of percentage of fluorescent cells were performed by using the Student's t test at the $p \leq 0.05$ level. The anti-6× histidine MAb-stained cells were stained with TO-PRO®-3 (Molecular Probes®, Life Technologies, Madrid, Spain), observed and photographed using a Leica DC100 confocal microscope (Leica Microsistemas S.L.U., Barcelona, Spain).

Injection of fingerling rainbow trout with rNV

Three different batches of fingerling rainbow trout (*O. mykiss*) of ~10 g each, free of IPNV and VHSV antibodies, were obtained from a local fish farm (Los Molinos, Madrid, Spain). Trout from different batches were pooled and maintained at 14–15 °C in 200-l aquaria with tap-dechlorinated carbon-filtered water provided with biological filters and fed with a commercial fish feed diet. After 2 weeks of acclimation, trout were separated into groups of six to eight trout per group and intraperitoneally injected with soluble rNV (10 μg), precipitated rNV (10 μg), polyH (2 μg, which is equivalent in moles to rNV) or PBS in a volume of 100 μl. Injected trout were then released to a 50-l aquarium per group and maintained at 14 °C. Two days after injection, trout were anaesthetised as described above. Pooled head kidney and spleen from each trout were extracted, immediately immersed in RNAlater (Ambion®, Life Technologies, Madrid, Spain) and maintained at 4 °C overnight before being frozen at −70 °C until processed. Ten micrograms of rNV per trout were intraperitoneally injected because this amount is ~10-fold higher than the amount of NV estimated in a VHSV-infected 10-g trout. The calculations were based on published estimations of number of active plaque-forming unit (pfu) per gram of trout (LaPatra et al. 1995), number of N molecules per pfu (Coll 1995), number of VHSV particles per pfu (Leong 1995), percentage of defective particles (Kim et al. 1999) and relative amounts of NV/N proteins in Western blots (Schütze et al. 1996). In addition, the NV/N ratio estimated by reverse transcription quantitative PCR (RT-qPCR) was 0.06, 2 days after intraperitoneal injection of 10^4 pfu of VHSV (not shown).

RNA extraction

Pooled head kidney and spleen from each individual trout were homogenised by using the Tissue Lyser Cell Disruptor (Qiagen Iberia, S.L., Madrid, Spain) 10 min at 50 Hz with 3-mm glass beads in RTL buffer. RNA was then extracted from the homogenates by using the RNAeasyPlus kit (Qiagen Iberia, S.L., Madrid, Spain) and eluted in RNase-free water. RNA concentrations were estimated by Nanodrop and the presence of 18 and 28S bands confirmed by denaturing RNA agar electrophoresis (Sigma-Aldrich Quimica SA, Madrid, Spain). Additional RNA quality control (RNA integrity number, RIN) was performed by NIMGenetics (Madrid, Spain). For each experimental group of six to eight trout, four of the best quality RNA (RIN>7.0) per group were chosen for microarray hybridisation. The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used was 4 (eight samples per slide), and all were hybridised simultaneously.

Design of oligo-microarrays enriched in rainbow trout immune-related genes (targeted microarrays)

The microarrays were enriched in rainbow trout immune-related genes as described previously (immune-targeted microarrays) (Ballesteros et al. 2012). The final 8x15K microarray corresponds to Agilent's ID032303 (Gene Expression Omnibus GEO platform submission number GPL14155). It contains 60-mer annotated probes each by duplicate, and different probes could detect the expression changes for each gene. To simplify the analysis of results, 1474 annotated probes were classified according to gene groups: VHSV-induced genes (VIG; number of probes, n=26), interferons (IFN) and its receptors (n=92), interferon-inducible proteins *mx* (MX; n=8), Toll-like receptors (TLR; n=32), complement components (CO, n=177), interleukins and its receptors (IL; n=120), macrophage-related genes (MA; n=126), tumour necrosis factor (TNF; n=33), cluster differentiation (CD; n=59) antigens, chemokines (CK) and its receptors (n=122), and caspases (8) (CASP) and transcription factors (TFs) (n=672). The trout microarray ID32303 used for these experiments was previously validated by RT-qPCR (Ballesteros et al. 2012). The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used was 4 (eight samples per slide), and all were hybridised simultaneously. Although to discover other possible non-immune genes targeted by rNV, a complementary DNA (cDNA) genome-wide microarray (e.g. 8x60K Salmonid from Agilent) could be more complete, we chose to focus on immune genes because there are no oligo microarrays available with such an enrichment of rainbow trout immune genes, there exist many non-annotated genes in available salmonid microarrays, and cDNA salmonid microarrays have higher crossreacting hybridisations and more qPCR is needed to validate the data. Our home-made rainbow trout oligo microarray used has more immune genes than any other available microarrays since it includes all the immune-related genes from the Agilent's EST-derived rainbow trout oligo microarray (ID16271) (Salem et al. 2008) as described before (Ballesteros et al. 2012).

Hybridisation of trout transcripts to the immune-targeted microarrays

Labelling of 2 μg of RNA (~50 μg/ml) and hybridisation to the microarrays were performed by NIMGenetics (Madrid, Spain) complying with the Minimum Information about a Microarray Experiment (MIAME) standards (Ballesteros et al. 2012).

Normalisations were performed by correcting the individual fluorescence in each microarray with the sum of all the fluorescent values according to the formula fluorescence of each probe/sum of all the probe fluorescence signals per microarray. The raw and normalised data were deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37330). After normalisation, outlier values (defined by those fluorescence values > or < mean±standard deviation per probe) were identified and eliminated (masked) from the calculations programmed in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA). Corrections with PBS (mock injected controls) values were first calculated for each probe as reported previously (Purcell et al. 2006) by using the following formula fluorescent value from rNV- or polyH-injected trout/mean fluorescent value from PBS-injected trout for obtaining rNV/PBS and polyH/PBS datasets, respectively. Final folds represented by rNV/polyH were then calculated by the following formula PBS corrected values from rNV-injected trout (rNV/PBS)/mean PBS-corrected value from polyH-injected trout (polyH/PBS). Means and standard deviations of the folds were then calculated (n=4). The two-tailed

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Student's t test statistic-associated p value was also computed for each probe. A double simultaneous criterion to define differentially expressed gene transcripts was used: (i) genes with folds >1.5 or <0.66 (drawn in the figures as negative values <−1.5) and (ii) folds deviated from the null hypothesis at $p \leq 0.05$.

Classification of rainbow trout genes in immune pathways and identification of genes common to many pathways (multipath genes)

To classify the rainbow trout genes of the ID032303-targeted microarray into immune-related pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/) and the WikiPathways (http://wikipathways.org/index.php/WikiPathways) pathway databases were used. Rainbow trout orthologous to human genes from available 74 immune pathways were manually searched on GenBank mRNA sequences (accessed from February to June of 2013). The list of the rainbow trout genes classified by pathways and their accession numbers were deposited in the GEO platform with submission number GPL17758. Each of the genes of the ID032303 microarray was then classified into the above-mentioned rainbow trout pathways by using a macro in Excel. To test for differentially expressed multipath genes, microarray genes were first classified into 74 rainbow trout immune-related pathways. In order to do that, the list of accession numbers of the microarray genes was first compared with the rainbow trout accession numbers obtained by manually searching their corresponding orthology in immune-related human pathways actually present in public databases. The classified genes were then systematically searched for genes (i) common to at least six immune-related pathways (Encinas et al. 2013), (ii) with differential expression folds <0.66 or >1.5 and (iii) significantly different from one of the 0.66/1.5 thresholds ($p \leq 0.05$). The pathways having more than five differentially expressed multipath genes were identified by using a program made in Origin Pro 8.6 (OriginLab Corporation, Northampton, MA, USA).

Results

Purification and yield of rNV

The protein extracts from recombinant *E. coli* coding for rNV were purified under denaturing conditions by His-tag affinity chromatography (IMAC) and eluted by imidazole yielding a single protein peak. Further chromatography on Sephadex G-100 at low pH and final dialysis against PBS at pH 7 produced abundant precipitates with only 4–7 % of the total rNV protein remaining in solution. Western blot analysis of soluble rNV with an anti-6× histidine monoclonal antibody (MAb) showed one band of ~17 kDa (Fig. 1). Numerous attempts to remove the amino terminal polyH tail of 33 amino acids were only capable of removing less than 55 % of the polyH tail (data not shown). Therefore, equimolecular amounts of a synthetic polyH peptide of 33 amino acids (4 kDa, MRGSHHHHHHGMASMTGGQQMGRDLYDDDKDRW) were injected also into trout as a control to derive differential expression data. Both resuspended-diluted precipitate and soluble supernatant were used to inject trout.

Effects on trout transcriptome modulation produced by rNV could not be due to LPS contamination since the amount in purified rNV was below 0.1 EU/ml.

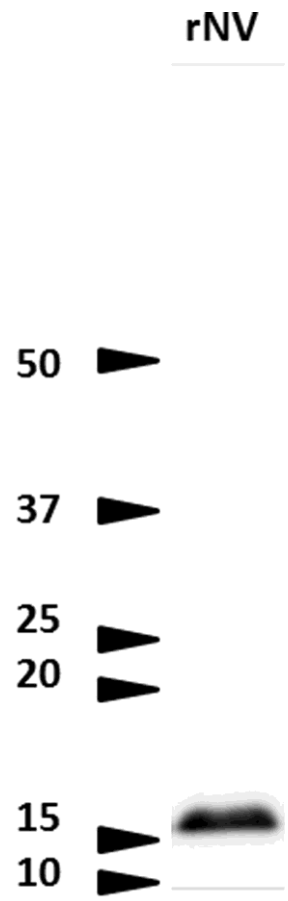

Fig. 1 Detection of rNV by Western blot. The nucleotide sequence of recombinant NV (*rNV*) derived from the VHSV-07.71 strain was cloned into the *E. coli* pRSETa plasmid. The rNV was purified in denaturing conditions by a Ni^{2+} affinity column and eluted with imidazole as indicated in 'Materials and methods'. Soluble rNV was separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and reacted with poly-6× histidine monoclonal antibody. The numbers to the left correspond to molecular weight standards in kilodalton

Selection of delivery route

Despite NV being an intracellular VHSV-expressed protein only in infected cells, extracellular NV should be also released after cellular lysis, as natural VHSV infection progresses from the point of infection. To study the possibility of extracellular NV being taken by some rainbow trout cells, head kidney cells were incubated in the presence of several concentrations of soluble rNV and its possible intracellular incorporation analysed by flow cytometry. Results demonstrated that 4–9 % of the cells in the P4 cell population (one of the populations containing 9.7±2.2 % of the total cells) and 2–5 % of the cells in P5 significantly incorporated extracellular rNV (Fig. 2a). The level of incorporation increased with rNV concentration, being highest at >50 ng/100 μl (Fig. 2b). Therefore, rNV could be injected into trout to allow for either soluble and/or macrophage-carried rNV to target internal immune-responsive organs such as head kidney and spleen. We chose intraperitoneal (ip) injection as the most practical delivery route to induce possible rNV effects on head kidney and spleen and because of the presence of peritoneal melanomacrophages in trout. Thus, head kidney and/or spleen cells isolated from fingerling trout ip injected with 1 μg of soluble rNV per gram of fish showed similar staining properties by flow cytometry analysis (Fig. 2c) than those obtained in the in vitro assays mentioned above. The ip injection of rNV could be a good approach for studying the NV effects on rainbow trout head kidney and spleen because VHSV naturally infects cells from these organs. Confirming the above-mentioned observations, the only cellular type which appeared with green fluorescence (detecting the presence of poly-6× histidines) at the confocal microscopy in both in vitro and in vivo experiments were typical melanomacrophages as demonstrated by their morphology. Melanomacrophages (n=50) took up rNV in their cytoplasm (Fig. 2d, fluorescence in green) rather than in its nuclei (Fig. 2d, fluorescence in blue) as shown by confocal microphotographs (Fig. 2c). Cytosolic location of rNV was previously observed in ZF4 and EPC-transfected cells with plasmid expressing His-tagged rNv and detected by fluorescence confocal microscopy (data not shown). No other cellular types showed green fluorescence.

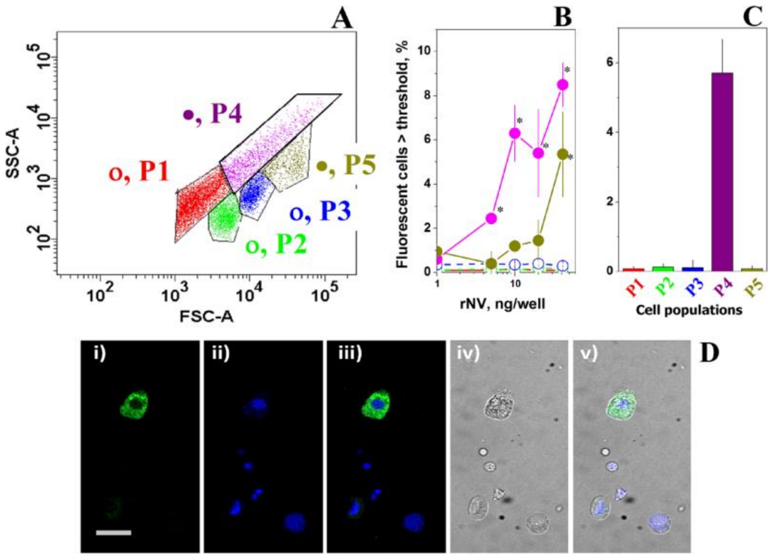

Fig. 2 Fluorescent cells in rainbow trout head kidney cell populations after in vitro incubation or in vivo injection of rNV. Head kidney cells were extracted from fingerling rainbow and separated by flow cytometry. **a** Five SSC/FSC cell populations were defined: *P1* in *red* (50.3±10.4 % of the total number of head kidney cells), *P2* in *green* (12.4±5.4 %), *P3* in *blue* (10.7±4.0 %), *P4* in *violet* (9.7±2.2 %) and *P5* in *dark yellow* (3.8±2.7 %). **b** Cells were plated (200,000 round cells per well) in a final volume of 100 μl in poly-D-Lys 96-well plates (Corning, NY, USA), soluble rNV added at different concentrations (1–50 ng/well) and plates incubated overnight at 14 °C before analysis. **c** Trout of ~1 g were injected with 1 μg of soluble rNV and 1 day later head kidney cells extracted. In both **b** and **c**, to detect intracellular rNV, cells were fixed to the plates, stained with anti-6× histidine MAb and suspended for flow cytometry by limited trypsin digestion (Chinchilla et al. 2013). **b**, **c** Distribution of the percentage of fluorescent cells among *P1–P5* cell populations. The number of fluorescent cells over a threshold containing 95 % (mean+2 standard deviations) of untreated cells was determined (Chinchilla et al. 2013; Ruiz et al. 2008). Mean and standard deviations were represented (n=2 for **b** and n=6 for **c**). *Significantly different from untreated cells by using the Student's t test at the p≤0.05 level. ○ *red*, P1.○ *green*, P2. ○, *blue*, P3. ●, *violet*, P4.●, *dark yellow*, P5. **d** Cytosolic location of rNV and cell morphology revealed by confocal microscopy. From *left* to *right*: green fluorescence locates rNV, detected by anti poly-histidine monoclonal antibody (*i*), blue fluorescence, TO-PRO-3-stained nuclei (*ii*), merged green and blue fluorescence (*iii*), phase contrast (*iv*) and merged all fields (*v*). *White horizontal bar*, 15 μm (colour figure online)

Overview of microarray results after intraperitoneal injection of rNV

To choose a suitable control to study the effects of rNV injection, the hybridisation to microarrays of RNA from head kidney and spleens from trout injected with polyH vs PBS was first studied. The analysis showed that 14.5 % of the probes were significantly expressed suggesting that injection of polyH had a small but measurable effect on trout transcript expression. Notwithstanding, this synthetic peptide did not induce the upregulation or downregulation of *mx*, *tlr*, *ifn* or *isg* genes, when compared with the PBS transcriptome profile. Therefore, polyH rather than PBS should be used to derive differentially expressed transcripts for polyH containing rNV.

The levels of transcription induced by the injection of soluble or precipitated rNV were each compared to PBS and then each compared to those caused by the injection of 2 μg polyH (corresponding to an equimolecular amount of that present in rNV). Most of the fluorescence signals from soluble rNV vs polyH-injected trout were <1-fold (down the black line in Fig. 3). In contrast, fluorescence signals from precipitated rNV vs polyH injected trout were more evenly distributed around fold 1 (not shown). Thus, the comparative analysis of both soluble or precipitated rNV vs polyH showed 52.8 or 8.5 % differentially expressed genes, respectively (data deposited in GSE 31557 and 37797), suggesting that rNV was required in soluble form to obtain maximal downregulation. The small amount of downregulated genes after injection of precipitated rNV provides a suitable specificity control for the soluble rNV effects. Therefore, soluble rNV was used to analyse more in detail differential expressions induced in each of the genes grouped in the categories described in 'Materials and methods'.

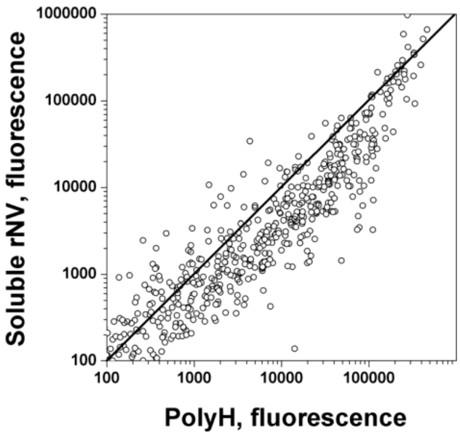

Fig. 3 Microarray fluorescence signals obtained from rainbow trout injected with soluble rNV. The figure shows the fluorescence signals of the transcriptomic profiles induced by soluble rNV vs polyH. Trout were injected with 10 μg of soluble rNV or 2 μg of polyH synthetic peptide (33 aa). The number of biological replicas was 4, one trout per replica. Head kidney and spleen were pooled from each individual trout. Each biological replica was hybridised twice (technical replicas). The total number of chips used were 4 (eight samples per slide) and all were hybridised simultaneously. After normalisation and outlier removal, the figure shows the fold means from four trout from each group. The fluorescence signals >100 arbitrary units (background fluorescence) were plotted. In each case, a *black straight line* has been drawn to show folds =1

Downregulation of IFN, myxovirus resistance and CASP by rNV. Novel and confirmatory results

The differential expression of selected genes known to be downregulated by wt-VHSV when compared to NV knockout VHSV such as interferon-related genes (i.e. type I interferons *ifn1-5* and interferon gamma, *ifng*) (De Kinkelin and Dorson 1973), *mx* (*mx1–mx3*) (Tafalla et al. 2007) and genes for caspases (Ammayappan and Vakharia 2011) was first studied. In this work, we have found multiple probes of type-1 interferon genes (*ifn1-5*) and *ifng* downregulated by rNV, confirming those previous reports. In addition, novel *ifn*-inducible genes (i.e. *gig2*, *iip2*, *iip30*, *ipf35*) and many *ifn* regulatory binding genes (*irf2*, *irf4*, *irf7*, *irf10*) were also downregulated (Fig. 4, IFN). Downregulation induced by rNV was similarly observed in all the myxovirus resistance GTPase genes (*mx1–mx3*) together with a gene for another GTPase regulator of G protein signalling (*rgs18*) (Fig. 4, MX). Caspase transcripts such as *casp1a*, *casp9* (initiator caspase) and *casp6* (effector caspase) were downregulated also by rNV injection (Fig. 4, CASP). Slight downregulation was found also for *casp3* (effector caspase). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of TFs

Downregulated genes by rNV among *stat* (signal transducer and activator) factors were *stat1* (involved in IFN-α/β and IFN-γ signalling pathways), *stat3* (negative regulator of COX2/prostaglandin E), and *stat5* (Fig. 4, TF). No expression data could be obtained for other *stat* transcripts since they have not been described in trout yet. Other downregulated genes were *nfkb* (p100/p52), *traf3* (tumour necrosis factor receptor-associated factor 3), *cebp* (ccaat/enhancer-binding protein), *atf1* (cAMP-dependent transcription factor), *jun* (proto-oncogen) and *tr* factor. Because the activating protein 1 (AP1, heterodimer composed by JUN, FOS, ATF1 family proteins), CEBP, STAT1 and TRAF2 proteins control the expression of many immune response genes (i.e. cytokines, interferons, interleukins, regulatory factors, etc), their gene downregulation should lead to a general inhibition of immune responses. The complete names of the genes shown in the figures have been included in the legends.

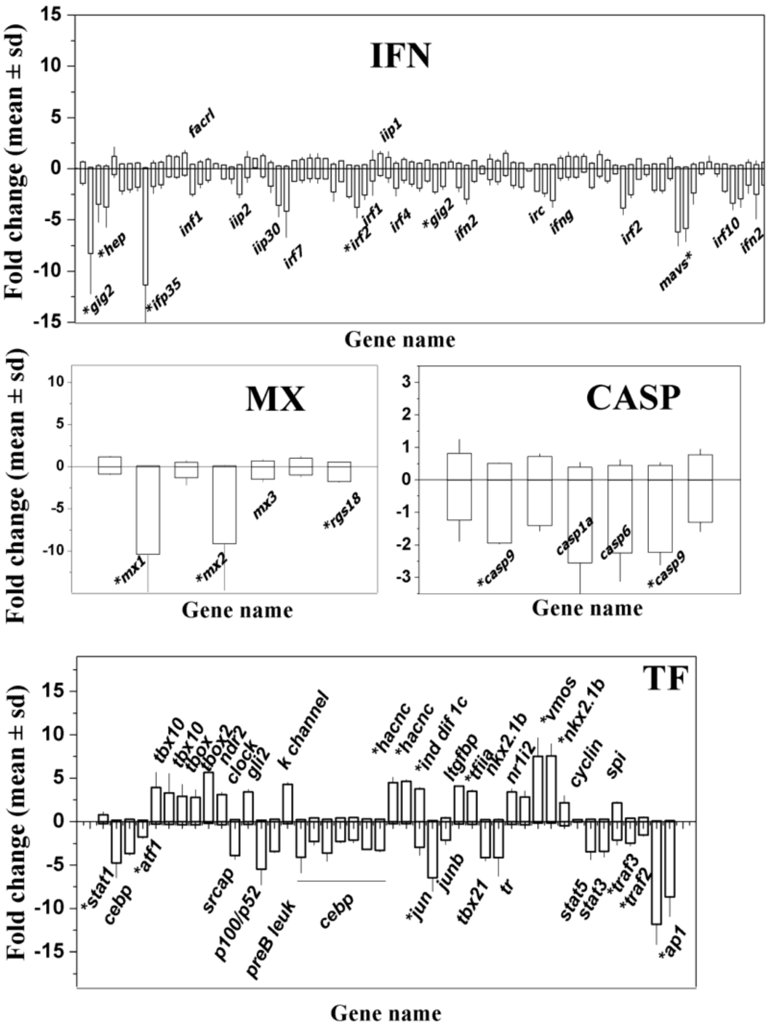

Fig. 4 Transcriptional expression changes induced by rNV among interferon (*IFN*) genes, myxovirus resistance genes (*MX*), caspase (*CASP*) genes and transcription factor (*TF*) genes. Differential fold changes±standard deviations are represented by bars (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. For each probe, differential expression folds were first calculated by comparing profiles from rNV or polyH-injected trout with respect to PBS-injected trout controls by the formula fluorescence from rNV or polyH injected trout/ mean fluorescence of PBS injected trout controls (n=4). Final folds were then calculated by the formula PBS corrected values from rNV-injected trout/mean PBS-corrected value from polyH-injected trout (n=4 trout). *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). IFN group, genes upregulated: *facrl*=interferon regulatory factor-like and *iip1*=interferon inducible protein 1 (AJ320156). Genes downregulated: *hep*=hepcidin, *gig2*=Tribbles homolog, G-protein-coupled receptor-induced gene 2 protein, *ifn1*=type 1 interferon 1, *ifn2*=type 1 interferon 2, *ifng*= interferon gamma, *ifp35*=interferon-induced protein 35, *iip2*=interferon inducible protein 2, *iip30*=interferon gamma inducible protein 30, *irc*= insulin receptor c, *irf1*=interferon regulatory factor 1, *irf2*=interferon regulatory factor 2, *irf4*=interferon regulatory factor 4, *irf7*=interferon regulatory factor 7, *irf10*=interferon regulatory factor 10 and *mavs*= mitochondrial antiviral signalling protein. MX group, genes downregulated: *mx1*=myxovirus resistance 1, *mx2*=myxovirus resistance 2, *mx3*=myxovirus resistance 3 and *rgs18*=regulator of G-protein signalling 18. CASP group, genes downregulated: *casp9*= caspase 9, *casp1a*=caspase 1a and *casp6*=caspase 6. TF group, genes upregulated: *clock*=circadian locomoter output cycles kaput protein, *cyclin*=cyclin, *gli2*=zinc finger transcription factor gli2, *hacnc*= hyperpolarisation-activated cyclic nucleotide-gate cation channel 2, *ind dif 1c*=inhibitor differentiation factor 1c, *K channel*=potassium channel tetramerisation domain, *ltgfbp*=latent transforming growth factor binding protein, *ndr2*=serine/threonine kinase 38 like, *nkx2.1b*=nk2 homeobox 1b, *nr1i2*=nuclear receptor subfamily 1, group I, member 2, *spi*=SPI-1 transcription factor, *tbox*=T-box containing transcription factor, *tbox2*=T-box 2 gene, *tbx10*=T-box 10 gene, *tfiia*=general transcription factor IIA and *vmos*=V-mos Moloney murine sarcoma viral onco-like prot. Genes downregulated: *ap1*=AP1 transcription factor, jun proto-oncogen, *atf1*= cAMP-dependent transcription factor ATF-1, *cebp*=CCAAT/enhancer binding prot, *jun*=jun proto-oncogen, *junb*=jun b proto-oncogen, *p100/ p52*=p100/p52 transcription factor (*nf-kb2*), *preB leuk*=pre-B-cell leukemia transcription factor protein 1, *srcap*=helicase SRCAP gene, *stat1*= signal transducer and activator of transcription 1, *stat 3*=signal transducer and activator of transcription 3, *stat5*=signal transducer and activator of transcription 5, *tbx21*=T-box 21 gene, *tr*=tissue factor, initiator of coagulation cascade, *traf2*=tnf receptor-associated factor 2 and *traf3*=tnf receptor-associated factor 3

Novel downregulation by rNV on the expression of VIG, TNF and TLR genes

Surprisingly, all VHSV-induced genes (*vig*) identified by subtractive hybridisation in previous works (O'Farrell et al. 2002), except *b191*, were significantly downregulated by rNV (Fig. 5, VIG). Most of the genes for inducers of inflammation known as members of the tumour necrosis factor (*tnf*) family were also downregulated by rNV, including several probes of *tnf10* (apoptosis inducer acting through *casp3* and *casp8*), *tnf11* (dendritic survival factor and activator of naïve T cells), *tnf14* (stimulator of apoptosis), *tnfa* (most important inducers of systemic inflammation), *ltb2*, *balm* and several *tnf* receptors (*tnfr*) (Fig. 5, TNF). Similarly, most of the genes for Toll-like receptors (*tlr*) were downregulated by rNV. Thus, gene expression of *tlr1* (receptor for microbial lipopeptides and glycolipids), *tlr3* (receptor for short-size dsRNA in membranes), *tlr22* (receptor described in zebrafish and catfish that senses long-size dsRNA) (Baoprasertkul et al. 2007; Zhang et al. 2013), *tlr7* (receptor for ssRNA) and *tlr9* (sensing CpG DNA) were downregulated (Fig. 5, TLR). In addition, negative regulators *tollip1* and *tollip2* were downregulated. Contrary to the gene *tlr5* for a membrane TLR form that remained unchanged, the gene for a soluble form was upregulated (Fig. 5, TLR). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of APM genes, CD antigens and IL

Most of the genes involved in antigen presentation machinery (APM) such as *tapbp* (codes for tapasin protein), *tap1* and *tap2* (transporter associated with antigen processing), *lmp2* (coding for a protein of the proteasome) and *b2m* (beta 2 microglobulin) were downregulated (Fig. 6, APM). Among the cluster differentiation (CD) antigens studied, not only downregulated but also upregulated genes were detected. Thus, some of the genes which were downregulated by rNV were *cd2* (implied in adhesion T cell-APC through CD58 protein), *cd3e* (part of the T cell receptor complex), *cd4* (T-helper cell marker), *cd8* (T-cytotoxic cell marker), *cd9* (thymocyte cell marker), *cd11* (dendritic cell marker, included in Fig. 7, MA), *cd28* (co-stimulatory gene expressed in T-cells), *cd80/86 m* (short mature form of a gene expressed in the membrane of antigen presenting cells), *cd79a* (associated with membrane-bound immunoglobulin in B-cells) or *cd200* (NK immunosuppressive cell surface gene) (Fig. 6, CD). In contrast, *cd33* (present in immature myeloid cells, possible Siglec-3 receptor), *cd97* (component of the immunoglobulin receptor of B cells, possibly related to decay accelerating factor of complement), *cd209* (marker of dendritic and macrophage cell subsets, which may increase phagocytosis) and *cd80/86 s* (soluble form of the gene) were upregulated (Fig. 6, CD).

Among the interleukin-related probes tested, there were also downregulated and upregulated genes. Thus, many of the interleukin genes (*il*) were downregulated, such as several probes of *il1b* (a lymphocyte mitogen produced by macrophages and an important mediator of inflammatory responses) and the gene for its receptor (*il1r*), *il2* (secreted by T cells), *il8* (a chemotactic factor produced by macrophages that attracts neutrophils, basophils and T-cells), *il12b* (maintenance of T helper responses to intracellular pathogens), *il15* (regulator of lymphoid homeostasis, effector of T cells and NK activation), *il17d* and its receptor (*il17r*). In contrast, other interleukin genes such as *il20* (regulator of proliferation/differentiation of keratinocytes), *il10* (an inhibitor of cytokines with anti-inflammatory effects), *il17a* and c (pro-inflammatory cytokine of activated T cells), *il18* and receptors like *il8r*, *il1r* or *il13r* (interleukin 13 (IL13), a pleiotropic cytokine involved in the regulation of IFNγ were upregulated (Fig. 6, IL). The complete names of the genes shown in the figures have been included in the legends.

Novel effects of rNV on the expression of macrophage cell markers (MA), CK and CO

Among the probes present in the microarray classified as macrophage-related genes, the genes for macrophage stimulating factor (*mcsf*), natural resistance macrophage α (*nrampa*), pre-B cell colony-enhancing factor (*pbef*), *cd11* (a dendritic cell marker) and cadherin (*cdh*) coding for an adhesive molecule (Fig. 7, MA) were among those downregulated by rNV. Only the genes for colony-stimulating factor 1 (*csf1*) and c-reactive protein (*crp*) were upregulated. Among the genes for chemoattractant cytokines or chemokines (*ck*) showing downregulated gene expressions after rNV injection were *ck1*, *ck2*, *ck3*, *ck5b*, *ck6*, *ck8b*, *ck10*, *ck12a*, *ccl4* (*scya4*), *cxc*, *cxcd1*, *cxcd2*, etc. Downregulation was also shown in some genes causing cytokine inhibition such as *socs1–socs7* (Fig. 7, CK). On the other hand, rNV induced upregulation of *ck4a*, *ck11*, *cxcr* and *ccr5* (Fig. 7, CK). The rNV downregulated many genes of the classic complement pathway, such as *c1q*, *cr1* (*c1* receptor), *c4* and perforin (*prf*). On the other hand, *bf1* and *h1* (inhibitors of complement) factors as well as some components of the final effector complex (*c5*, *c7*, *c8*, *c9*) were upregulated by rNV (Fig. 7, CO). The complete names of the genes shown in the figures have been included in the legends.

Identification of regulated genes common to several pathways (multipath genes) suggests functionally important NV targets

Because the above-mentioned analyses of microarray data were mainly descriptive, we analysed the differentially expressed genes based on their presence on rainbow trout/human orthologous immune pathways (multipath genes). Thus, the numerous immune-related genes downregulated by rNV suggested that a few master genes targeted by NV could be responsible. Those candidates could be amongst multipath genes since they would interconnect several pathways and therefore have the widest impact. Differentially expressed multipath genes filling the above-mentioned criteria were thus identified (Table 1). Among all genes downregulated by rNV, there are three (*stat1*, *jun* and *atf1*) whose transcription factors are present in ≥20 immune-pathways and in some of the pathways containing the highest numbers of multipath genes such as for TLR (n=9 multipath genes), interferon type I (n=6), MAPK (n=6) and RIG-I (n=6). All the rest of the differentially expressed multipath genes identified were also downregulated

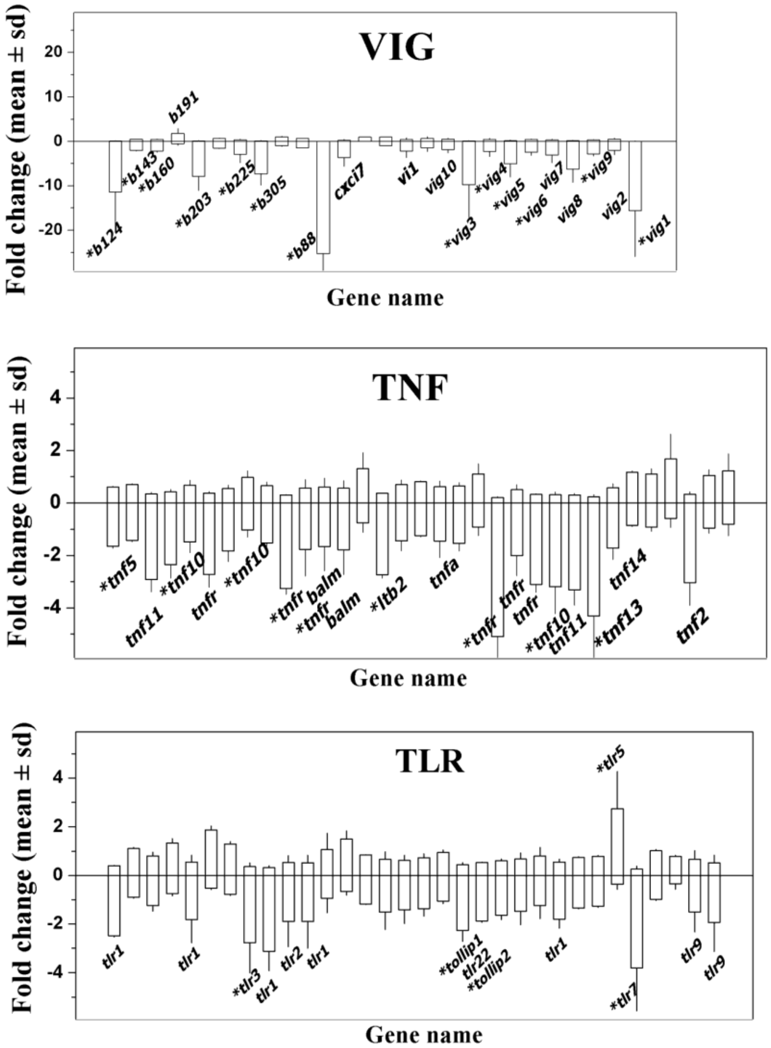

Fig. 5 Transcriptional expression changes induced by rNV among VHSV-induced (*VIG*), tumour necrosis factor (*TNF*) and Toll-like receptor (*TLR*) gene groups. Differential fold changes±standard deviations are represented by *bars* (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant (p≤0.05) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. VIG group, genes upregulated: *b191* (AF483535). Genes downregulated: *vig-1* (AF076620), *vig-2* (AF290477), *vig-3* (AF483529), *vig-4* (AF483530), *vig-5* (clone B17), *vig-6* (clone B126), *vig-7* (AF483527), *vig-8* (clone B68), *vig-9* (AF483533), *vig-10* (AF483534), *b203* (AF483538), *b143* (AF483539), *b225* (AF483540), *b88* (AF483541), *b160* (AF483545), *b124* (AF483546), *b305* (AF483542), *cxci7*=VHSV-induced protein 7 (*vig7*) and *vi1*=VHSV-induced protein 1. TNF group, genes downregulated: *balm*=BAFF and APRIL-like molecule, *ltb2*= lymphotoxin beta 2, *tnf10*=tumour necrosis factor superfamily 10 (TNFSF10), *tnf11*=tumour necrosis factor superfamily 11, *tnf13*=tumour necrosis factor superfamily 13, *tnf14*=tumour necrosis factor superfamily 14, *tnf2*=tumour necrosis factor superfamily 2, *tnf5*=tumour necrosis factor superfamily 5 (CD40), *tnfa*=tumour necrosis factor alpha and *tnfr*=tumour necrosis factor receptor. TLR group, genes upregulated: *tlr5*=Toll-like receptor 5. Genes downregulated: *tlr1*=Toll-like receptor 1, *tlr2*=Toll-like receptor 2, *tlr3*=Toll-like receptor 3, *tlr7*=Toll-like receptor 7, *tlr9*=Toll-like receptor 9, *tlr22*= Toll-like receptor 22, *tollip1*=Toll-interleukine I receptor interacting protein 1 and *tollip2*=Toll-interleukine I receptor interacting protein 2

after rNV injection (*traf3*, *traf2*, *tnfa*, *ifng*, *casp6*, *irf1*, *il1b*, *irf10*, *socs1*, *ifn1*, *irf2*) (Table 1).

Discussion

In the present work, we have determined the trout transcriptome changes induced by the recombinant NV (rNV) protein from VHSV after 48 h post-intraperitoneal injection. Previous published results showed that a natural VHSV infection on EPC cells induced the highest NV expression level at 48 h post-infection (hpi) (Kim and Kim 2012). Therefore, we decided to study the transcriptome changes at 48 h as the best approach to the natural conditions of infection. NV effects might be different on gene transcriptome modulation along time. However, we have taken two considerations: the highest NV expression at 48 hpi (Kim and Kim 2012) and possible half-life of rNV within cell. In mammals, GFP protein has half-life of ~26 h (Corish and Tyler-Smith 1999) and the precursor IL1β is degraded with a relatively short half-life of 2.5 h (Moors and Mizel 2000). Therefore, it is probable that

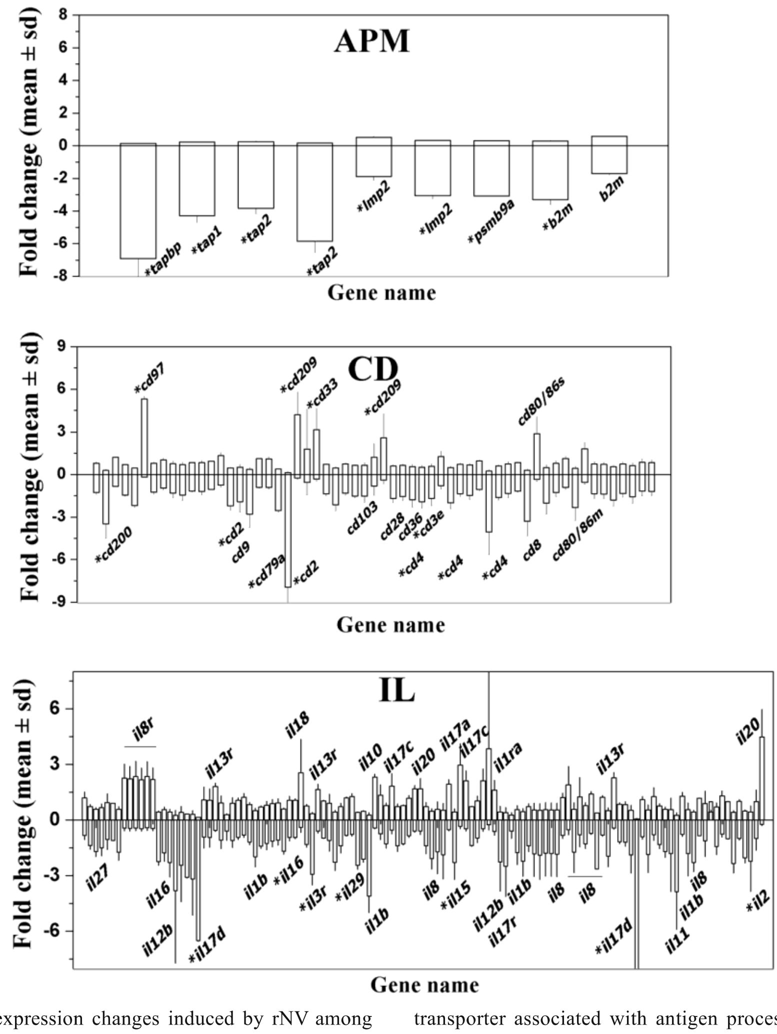

Fig. 6 Transcriptional expression changes induced by rNV among antigen presentation machinery (*APM*), cluster of differentiation (*CD*) and interleukins and their receptors (*IL*) gene groups. Differential fold changes±standard deviations are represented by *bars* (Y-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. APM group, genes downregulated: *tapbp*=tapasin, TAP binding protein, *tap1*=transporter associated with antigen processing 1, *tap2*=transporter associated with antigen processing 2, *lmp2*=*psmb9*, *psmb9a*=proteasome subunit, type 9, *b2m*=beta-2 microglobulin. CD group, genes upregulated: *cd97*, *cd209*, *cd33*, *cd209* and *cd80/86 s* (soluble form). Genes downregulated: *cd200*, *cd2*, *cd9*, *cd79a*, *cd103*, *cd28*, *cd36*, *cd4*, *cd3e*, *cd8* and *cd80/86 m* (membrane form). IL group, *r* means receptor. Genes upregulated: *il1ra*, *il8r*, *il10*, *il13r*, *il17a*, *il17c*, *il18* and *il20*. Genes downregulated: *il2*, *il3r*, *il8*, *il11*, *il12b*, i*l15*, *il16*, *il17d*, *il17r*, *il1b*, *il27* and *il29*

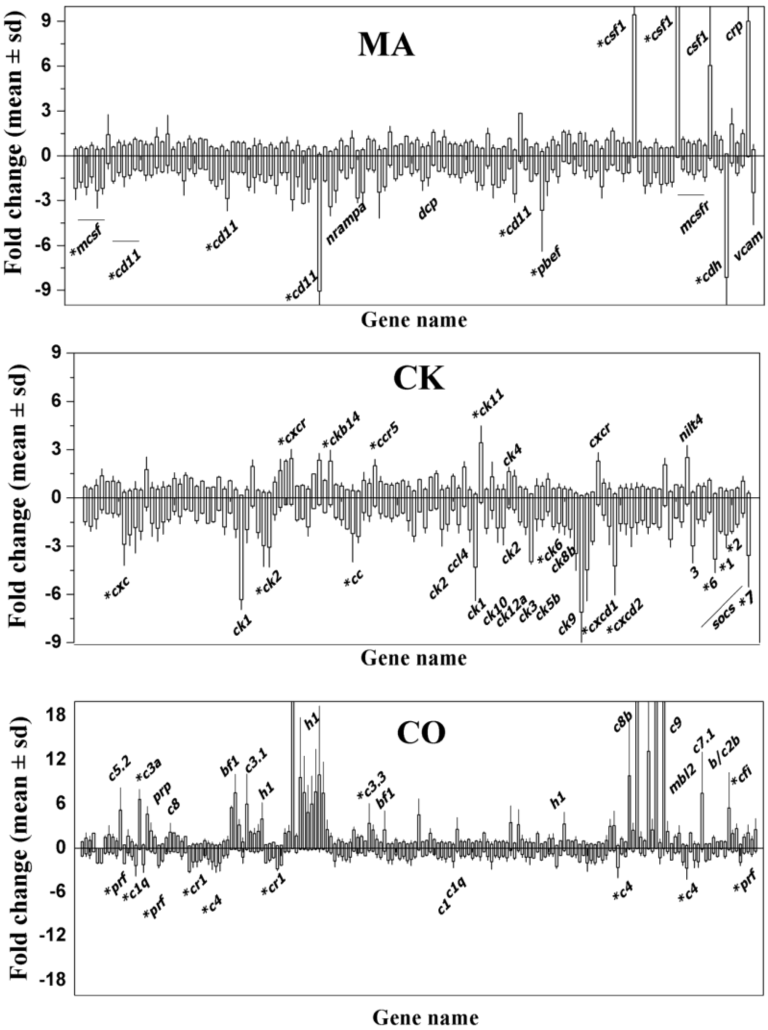

Fig. 7 Transcriptional expression changes induced by rNV among macrophage-related (*MA*), chemokines (*CK*) and complement components (*CO*) gene groups. Differential fold changes±standard deviations are represented by *bars* (*Y*-axis). Probes defining distinct genes are represented in *X*-axis. *Names in the bars* represent folds >1.5 (+, positive upregulated values) and <0.66 (−, negative downregulated values). Those folds statistically significant ($p \leq 0.05$) are represented by an *asterisk* (*). Fold change calculations are described in 'Materials and methods' and Fig. 4 legend. MA group, genes upregulated: *csf1*=colony stimulating factor 1, *crp*=c-reactive protein. Genes downregulated: *mcsf*=macrophage conolony stimulating factor, *cd11*=alpha chain of many integrins, *nrampa*=natural resistance macrophage alpha, *dcp*=dendritic cell protein, *pbef*=colony pre-B enhancing factor, *mcsfr*=macrophage colony stimulating factor receptor, *cdh*=cadherin and *vcam*=vascular cell adhesion molecule-1. CK group, genes upregulated: *cxcr*=cxc receptor, *ckb14*=cc-chemokine 14, *ccr5*=cc-chemokine 5 receptor, *ck11*=cc-chemokine 11, *ck4a*=cc-chemokine 4a and *nilt4*=novel immunoglobulin-like transcript 4. Genes downregulated: *cc*=β-chemokines, *ccl4*=cc-chemokine, *ck1*=cc-chemokine 1, *ck10*=cc-chemokine 10, *ck12a*=cc-chemokine 12a, *ck2*=cc-chemokine 2, *ck3*=cc-chemokine 3, *ck5b*=cc-chemokine 5b, *ck6*=cc-chemokine 6, *ck8b*=cc-chemokine 6, *ck9*=cc-chemokine 9, *cxc*=α-chemokines, *cxcd1*=cxc d1 chemokine, *cxcd2*=cxc d2 chemokine, *socs1*=suppressor of cytokine signalling 1, *socs2*=suppressor of cytokine signalling 2, *socs3*=suppressor of cytokine signalling 3, *socs6*=suppressor of cytokine signalling 6 and *socs7*=suppressor of cytokine signalling 7. CO group, genes upregulated: *c5.2*=complement component 5, isoform 2, *c3a*=complement component 3a, *c9*=complement component 9, *prp*=properdin, *c8*=complement component 8, *bf1*=complement factor B, *c3.1*=complement component 3, isoform 1, *h1*=complement factor H1, *c3.3*=complement component 3, isoform 3, *c8b*=complement component 8b, *mbl2*=mannose-binding lectin, *c7.1*=complement component, isoform 1, *cfi*=complement factor I and *b/c2b*=complement factor B/C2b. Genes downregulated: *prf*=perforin, *c1*=component, *c1q*=component of C1 (classical complement activation pathway), *cr1*=complement receptor 1 and *c4*=complement component 4

to carry out studies of the trout transcriptome at times longer than 48 h post-injection of NV could not show real NV effects. Our main results showed that rNV induced an inhibition of multiple innate and adaptive genes. In the majority of organisms, innate immune response starts immediately upon host exposition to antigen and extends for at least 72 h, whereas the adaptive immune response begins close to 72–96 h, later than the innate. The effect induced by rNV on transcriptome showed the downregulation (and some upregulation) of multiple genes involved in the innate and adaptive immune response, such as those participating in antigen presenting (*tap*, *lmp2*, *β2m*, *cd4*, *cd8*) and other macrophage-related genes. Some downregulated interleukins, interferons and transcription factors activated by innate immunity are involved in triggering adaptive immune responses. This could explain why the adaptive immune genes are also inhibited at 48 h post-NV treatment. The NV protein induced a loss of innate immune response and a delay of adaptive immune response, which will favour viral replication at least during the early stages of VHSV infection under a weak antiviral state. To our knowledge, this is the first report suggesting most important possible rNV targets and describing extensive transcriptional in vivo profile changes induced by the rNV protein in any fish novirhabdovirus.

Identification of *stat1* and *jun/atf1* as differentially expressed multipath genes

By using microarray data and rainbow trout immune pathways, the transcription factors *stat1* and *jun/atf1* have been identified as the downregulated multipath genes more represented in the pathways studied (>25 % of the immune pathways, Table 1). The potential of multipath genes to suggest functional studies had been used previously to investigate the effects of spring viraemia virus (SVCV) infection on zebrafish to suggest prevention of drug candidates (Encinas et al. 2013). The multipath analysis also suggested other multipath genes which were differentially expressed after rNV injection and were upstream or related to either *stat1* (i.e. interferon-related genes such as *ifng*, *ifn1*, *irf1*, *irf2*, *irf10*, *socs1*, *casp6*, *il1b*) or *jun/atf1* (i.e. tumour necrosis factor-related genes such as *tnfa*, *traf2*, *traf3*).

The mechanisms of NV action could involve the inhibition of some downregulated genes for multipath TFs found such as *stat1* (and *jun/atf1*). Our recent prelimary experiments indicated that rNV and STAT1 might interact, although further experiments need to be carried out to affirm that interaction. Signal transducer and activator of transcription (STAT1) is a cytoplasmic latent transcription factor that becomes activated by various extracellular stimuli, including IFNγ and IFN1 after binding to their specific cell surface receptors. Mammalian phosphorylated homodimers of STAT1 bind to interferon-gamma-activated sequence (GAS), while STAT1 heterodimers bind to interferon-stimulated response element (ISRE) transcription factor binding sites (TFBS). Binding to those TFBS leads to an increased expression of many *ifn*-stimulated

Table 1 List of differentially expressed genes which were present in at least six immune-related pathways ranked by the number of pathways

Short name	Accession number	Gene description	No. of paths	NV Mean	±SD	N
stat1	NM_001124746.1	Signal transducer/activator	27	**−4.73***	1.75	16
jun	NM_001124411.1	Jun proto-oncogen	25	**−2.94***	0.95	4
atf1	NM_001124559.1	cAMP-dependent transcription factor	20	**−1.75**	0.19	8
traf3	NM_001124615.1	Tumour necrosis factor receptor 3	14	**−2.10***	0.61	4
traf2	NM_001124393.1	Tumour necrosis factor receptor 2	13	**−1.52**	0.18	4
tnfa	AJ277604.2	Tumour necrosis factor alpha	12	**−1.45**	0.63	12
ifng	NM_001124620.1	Interferon gamma	11	**−3.11***	0.71	20
casp6	NM_001124271.1	Caspase 6	9	**−2.24***	0.89	4
irf1	NM_001124293.1	Interferon regulatory factor 1	9	**−3.80***	1.02	24
il1b	AJ223954.1	Interleukin-1-beta	8	**−4.10***	0.90	12
irf10	AJ829672.1	Interferon regulatory factor 10	8	**−2.39**	1.13	4
socs1	NM_001146166.1	Suppressor cytokine signalling 1	8	**−2.08***	0.13	4
ifn1	AM489418.1	Type I interferon 1	6	**−2.52***	0.21	28
irf2	NM_001124438.1	Interferon regulatory factor 2	6	**−1.80***	0.17	8

Differentially expressed genes with folds <0.66 (downregulated, -1/fold) present in >5 pathways (multipath genes) of the 74 screened together with their corresponding mean folds were filtered/extracted from the microarray data. The differentially expressed multipath genes were tabulated together with their corresponding mean folds (bold) and standard deviations by comparing rNV-PBS vs polyH-PBS. The genes were then ordered by the number of pathways in which they were present. Other genes common to >6 pathways were not differentially expressed, for instance, *p53* (NM_001124692.1, common to 16 pathways), *tnf6* (TC121157, 13), *il6* (NM_001124657.1, 11), *lck* (NM_001124542.1, 11), *mhc2.daa* (AJ251431.1, 10), *junb* (NM_001124520.1, 10), *if2a* (NM_001124296.1, 9), *myc* (NM_001124699.1, 9), *tgfb* (X99303.1, 9), *il12b* (AJ548830.1, 8), *il8* (AY160981.1, 7), *grb* (AY173044.1, 7) and *irak4* (FN598575.1). The *atf*, *traf2* and *tnfa* genes have been included because of their relationships to other genes in the table

"–" downregulated, *n* total number of probes per gene analysed

*Significantly different from <0.66 (<−1.5) at the $p \leq 0.05$ level genes (*isg*) that contain those TFBS sequences in their promoters, closing the regulatory circle (IFNs-STAT1-IFNs). *Socs1* (another multipath gene) encodes a STAT1-inducible feedback inhibitor of IFN signalling. Other multipath genes downregulated by rNV, such as *il1b* or *casp6*, are also related to *stat1*. On the other hand, the products of *jun/atf1* belong to transcription factors participating in the activating protein 1 (AP-1) heterodimer which targets tumour promoter antigen (TPA) on the DNA and have many immunological effects including those related to *tnf* and *tnf*-related genes (another multipath genes downregulated after rNV injection, Table 1) (Manicassamy and Pulendran 2009; Parker et al. 2007; Schröder et al. 2006; Schröder and Bowie 2007; Takeuchi and Akira 2007; Thompson and Iwasaki 2008). The interferon system has a key role in controlling viral infections. Different kinds of viruses (RNA, DNA) have developed strategies to circumvent the IFN responses. Mammalian rhabdoviruses such as vesicular stomatitis virus (VSV) and rabies virus (RV) use the matrix protein M (M_{VSV}) and the phosphoprotein P (P_{RV}), respectively, to inhibit nuclear accumulation of phosphorylated STAT1, of binding of phosphorylated STAT1 to *isg* genes (Chelbi-Alix et al. 2006; Vidy et al. 2007). Among fish rhabdoviruses, SVCV lacks the NV gene, being NV restricted to the genus *Novirhabdovirus*, whose member VSHV has maintained the NV protein for inhibiting the host antiviral state.

Immune gene regulation induced by rNV

Microarray data were obtained from trout spleen and head kidney. Regarding the rNV delivery route, because head kidney trout cells were capable of incorporating extracellular rNV in vitro as well as in vivo (see results), intraperitoneally injected rNV might have been internalised to the cytosol of intraperitoneal macrophages and then transported to head kidney and/or spleen. Transcriptome changes might reflect either those taking place in the macrophage or those induced by proteins released from the rNV-containing macrophages in other cells in the internal organs. The possibility that the observed changes in gene expression could be due to changes in cell type abundance in head kidney and spleen due to cell migration rather than within constant cell populations (i.e. macrophages) must be also considered as a possible alternative mechanism to explain the profiles of differential expression. Future histological and/or complementary flow cytometry analysis of head kidney and spleen will be used to study likely rNV effects on cell-type abundances (ongoing work).

On the other hand, we wanted to investigate whether the precipitated rNV also had any effect on the gene expression of the immune-related genes, noting that precipitated NV had no effect (results not shown). Expression changes modulated by soluble rNV could be observed in constitutive and non-constitutive expressed genes. This might be due to manipulation and injection (PBS or rNV) of all fish leading to certain stress and subsequent upregulation of the gene expression (e.g. *mx* and *ifns*). Previous works describe that the *ifn1* gene is not constitutively expressed in rainbow trout whereas *ifn2* and *ifn3* are (Zou et al. 2007). However, other works showed a constitutive expression of many isoforms of all of them (Purcell et al. 2009). In the present work, all the trout groups injected with PBS or rNV might be a quiet initial upregulation, but only in the rNV-injected group, a downregulation is observed due to rNV effect, being relative values in the comparison rNV vs PBS.

Immune-targeted microarray whole data (spleen and head kidney) confirmed previous results on *ifn*-related and *casp* genes, and this increases the confidence in the rest of the novel data described in this work. We have characterised novel downregulated *ifn*-related genes also amongst the multipath genes (*ifn1*, *ifng*, *irf1*, *irf2*, *irf10*). The downregulation in the caspases confirmed also that rNV induces an anti-apoptotic state in the infected cells to maintain VHSV replication, as suggested by previous functional studies comparing wt- and NV knockout novirhabdoviruses (Ammayappan and Vakharia 2011).

One of the most characteristic new observations came from the VHSV-induced genes (*vigs*). These genes were previously identified by others as in vitro early responses characteristic of VHSV-infection by using subtractive suppressive hybridisation of RNAs isolated from infected head kidney rainbow trout leukocytes (O'Farrell et al. 2002). Upregulation of *vigs* was demonstrated by semiquantitative RT-qPCR and confirmed by Northern blot analysis. In vivo downregulation of most *vigs* by rNV has been described for the first time here, suggesting that one of the most significant effects of rNV is to suppress those early host responses, thus highlighting the importance of *vigs* for natural fish immunity towards novirhabdoviruses.

Similarly, most of the rainbow trout *tnf*-related (inducers of inflammatory responses) and most of the *tlr*-related genes were downregulated by rNV, indicating that rNV inhibits inflammation and detection of viral RNA such as *tlr3* (dsRNA) and *tlr7* (ssRNA). Contrary to other *tlrs*, *tlr5* is found upregulated. We cannot exclude a previous bacterial impact on trout. However, this should be reflected among all trout whatever rNV or polyH they were injected. We think that an explanation for *tlr5* upregulation might be because of the different probe binding to its complementary sequence due to a not yet described polymorphism. In this work, *tlr5* gene expression was only defined with one probe located at the 3′ end untranslated region in its mRNA. Single nucleotide polymorphisms (SNPs) within *tlr5* coding regions have been found in humans, pigs, cattle, sheep and other species (Hawn et al. 2003; Smith et al. 2012; Yang et al. 2013). However, little is known about SNPs in mRNA-untranslated regions.

Since rNV induced downregulation of antigen presentation machinery (APM) genes and since their corresponding proteins have a major role in processing antigenic proteins into peptides to charge MHC class I molecules for antigen presentation to CD8$^+$ T-cytotoxic lymphocytes, rNV could impair these host mechanisms for appropriate processing of pathogen antigens (i.e. those resulting from VHSV infection), delaying the beginning of adaptive immune response.

The cluster differentiation (*cd*) genes downregulated by rNV included the most characteristic T-helper and T-cytotoxic *cd4* and *cd8* markers, respectively, suggesting also downregulation of the beginning of adaptive immune responses. Another example of adaptive immune signalling interference by rNV was downregulation of *cd28*, similarly to what has been reported in mammalians with the NEF protein of HIV-1 (El-Far et al. 2013). Other data on *cds* might indicate that rNV also stimulates immune responses like any other protein antigen, which could have been injected, for instance, downregulation of *cd200* (NK immunosuppression) and upregulation of *cd97*, *cd209*, *cd33*, etc.

There was an extensive interleukin (*il*) upregulation and downregulation induced by rNV which might have extensive implications for the immune response. Thus, downregulation of *il1b* and *il8* could cause downregulation of *nfkb* and therefore hamper the immune responses in many other pathways. Regarding *il17*, while *il17a* and *il17*c were upregulated by rNV, *il17d* (affecting *il1b* and *tnfa*) was downregulated by rNV. The transcripts of *Il17a* are produced in multiple cell types including CD4+αβ, γδ T-cells, natural killers and neutrophils, and IL17a protein is an inducer of granulocyte/monocyte stimulating factor (*gcsf*), several chemokines and neutrophil recruitment factors (Kolls and Linden 2004). In mouse, the *il17a* gene was upregulated by *il23* in *soc3* (activation of macrophages)-deficient mice (Aggarwal et al. 2003). In those conditions, IL23 induced STAT3 phosphorylation, which in turn increased *il17a* gene transcription. Therefore, *il17a* upregulation in trout may be caused by downregulation of *soc3* by rNV.

Downregulation of *nramp*, *pbef* and *cd11* by rNV might have important consequences for the responses of macrophages to VHSV infection and/or to the carry-over of NV by macrophages. The *nramp* gene family codes for macrophage membrane proteins related to phagocytosis (Govoni and Gros 1998), while the protein encoded by *pbef* is an inhibitor of apoptosis (by inhibiting CASP3 and CASP8) (Jia et al. 2004) and *cd11* encodes for an important dendritic cell marker. Therefore, downregulation/upregulation of these genes by rNV suggests their effects on inhibition of phagocytosis, infection-defensive apoptosis and antigen processing and presentation.

Some chemokine (*ck*) gene modulations were similar to those previously reported in trout fins after bath infection with VHSV (i.e. upregulation of *ck11* and downregulation of *ck10* or *ck12*) (Montero et al. 2011) or epidermis (*ck6* and *ck9*). The rNV upregulation of *inos* might favour VHSV replication since increase in nitric oxide (NO) described in turbot macrophages infected with VHSV showed that NO had no antiviral effects (Tafalla et al. 1999). Other differential expression of cytokines remains to be explained. There are no reported studies with both *socs1* (involved in T_{h} and T_{reg} regulation) (Lu et al. 2009; Yoshimura et al. 2012) and *socs3* (Liu et al. 2008) being downregulated by any other viral protein.

Antibody recognition at the beginning of the classical complement pathway seems to be inhibited by rNV by downregulation of *c1q* similarly to *prf* (perforin) implicated at the end in membrane pore formation. In contrast, *h1* (an inhibitor of complement) and most genes implicated in the final attack complex (*c5*, *c7*, *c8*, *c9*) were upregulated. These results suggest that rNV modulates complement components to delay any possible antibody and/or cellular-mediated responses and that host responses try to maintain some complement components activated. This scenario might reflect the host/pathogen fight at the beginning of VHSV infection.

In vivo protection had been reported for VHSV challenge with NV knockout VHSV in flounder (Kim et al. 2011) and for IHNV challenge with NV knockout IHNV for trout (Thoulouze et al. 2004) as live-attenuated vaccine candidates in a dose-dependent manner (Kim et al. 2011; Thoulouze et al. 2004). Data presented in this work could explain much better than before the protection induced by knockout novirhabdoviruses as due to the lack of an extensive inhibition of host early immune gene responses. Forthcoming studies will be focused on the characterisation of molecular targets for NV (e.g. *stat1*, *atf1/jun*, *nfκβ*). The results described in this work, novel in the use of rNV in vitro/in vivo and of immune-targeted microarrays, produced a better understanding of additional possible NV functions and suggested some working hypothesis to continue the work on NV effects. The novel data obtained improves our knowledge of the immunosuppression mechanisms used by VHSV, and similar strategies could be used with other novirhabdoviruses. The results of this work also open up the possibility to use rNVs as a new tool to further study immune response mechanisms in other teleost fish.

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PAPER II: *Functional study of VHSV NV in ZF4 cells*

IDENTIFICATION OF THE FUNCTIONAL REGIONS OF THE VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV) NV PROTEIN: VARIANTS THAT IMPROVE FUNCTION

El gen NV está presente únicamente en las cuatro especies que componen el género *Novirhabdovirus*: IHNV, VHSV, HIRRV y SHRV. A pesar de la escasa homología inter-especie que comparten las proteínas NV, todas ellas (a excepción de la NV de SHRV) mantienen una función común encaminada a favorecer la replicación de los distintos virus. El objetivo principal de este trabajo era determinar y estudiar las regiones de la proteína NV de VHSV implicadas en su función. Además se estudió la implicación de una región concreta de la NV de VHSV en base a su homología con la NV de IHNV, dado que dicha región de la NV homóloga de IHNV tiene la función de transportar la proteína al núcleo para ejercer su función. También, se intentó establecer una correlación entre la localización subcelular y la función de la NV de VHSV, y así compararla con las proteínas NV de otros novirhabdovirus. Para ello, se ensayaron ocho fragmentos que cubrían diferentes secuencias lineales de la NV. Además, se generaron once variantes de NV con cambios únicos de sus aminoácidos correspondientes por alanina en las posiciones 28, 31 y de la 33 a la 41. La funcionalidad de estos fragmentos y variantes se estableció midiendo los niveles de expresión de transcritos de *mx* e *il8* en células ZF4 transfectadas con las construcciones de NV mencionadas. De los fragmentos ensayados, el fragmento N-terminal (F1) conservaba en mayor medida la función de inhibición de *mx* e *il8*, como la proteína NV completa. Por otro lado, el fragmento C-terminal (F2) perdía esta función porque aumentaba los niveles de *mx* e *il8*. Por su parte, las variantes NV36, NV39 y NV41 mostraron una función de inhibición incrementada respecto a la proteína NV nativa. La localización subcelular de todas las variantes fue principalmente citoplásmica, al igual que ocurre con la NV nativa. Asimismo, el fragmento N-terminal que preservaba la función también tenía una localización citosólica. En conjunto, estos datos sugieren que la secuencia de la parte N-terminal de la NV de VHSV se correlaciona con una correcta localización y, a su vez, con la función. Además, estos resultados se compararon con la localización de las proteínas NV de otros novirhabdovirus (IHNV, HIRRV), estableciéndose homologías y diferencias entre ellas. El mapeo funcional de la NV de VHSV contribuye al estudio de la interacción de la NV de VHSV con su ligando PPM1Bb, para poder desarrollar futuros inhibidores o activadores del mismo. Finalmente, se pone de manifiesto que los polimorfismos de la NV pueden tener gran relevancia en la patogenicidad del virus (VHSV).

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Full length article

Identification of the functional regions of the viral haemorrhagic septicaemia virus (VHSV) NV protein: Variants that improve function

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ABSTRACT

Non-virion (NV) protein is essential for an efficient replication increasing the pathogenicity of the *Salmonid novirhabdovirus* (formerly IHNV), *Piscine novirhabdovirus* (formerly VHSV), and *Hirame novirhabdovirus* (HIRV). The interferon system, apoptosis, and other immune-related genes are modulated by NV to finally induce a deficient antiviral state in the cell. However, little is known about the VHSV NV regions involved in function and location. Here, eight different NV 07.71 fragments and eleven NV 07.71 mutants derived from the region between the two first α-helices have been studied in order to establish the *mx* and *il8* transcript levels in ZF4 cells and the subcellular location. As a result, we determined that the N-terminal part of NV preserves the same ability as the wild-type (wt) NV in *mx*/*il8* modulation and it also shares the subcellular location. Among NV mutants, some induced *mx* upregulation (N34A, C35A, D38A, and S40A) but maintained the *il8* levels stable when compared to wt-NV in ZF4. Four NV mutants (D28A, N31A, L33A, and F37A) were not affected by the mutation and showed *mx* and *il8* transcript levels similar to wt-NV. Surprisingly, mutants D36A, R39A, and D41A induced a stronger downregulation of both *mx* and *il8* transcript levels than wt-NV, suggesting that a more stable structure and an improved interaction with ligands could be achieved through these mutations. Amino acids at positions 36 and 39 are conserved among known VHSV NV proteins whereas at position 41 two different amino acids have been described. To date, no natural NV proteins with alanine at positions 36, 39, and 41 have been found. In addition, wt-NV, all NV mutants, and one N-terminal NV fragment were located at cytoplasm with a characteristic pattern, which might support that cytoplasm is the site for interaction with candidate ligands such as PPM1Bb. Taken together, the data presented in this work indicated that NV function relies on the first part of the molecule and is dependent on tertiary structure rather than on the linear one. This study could lead to a better knowledge of VHSV escape from fish antiviral mechanisms as well as to future studies on immune targets.

1. Introduction

The *Novirhabdovirus* genus was defined within the *Rhabdoviridae* family solely due to the presence of the non-virion (NV) gene. The four species of the genus are *Salmonid novirhabdovirus* (formerly IHNV), *Piscine novirhabdovirus* (formerly VHSV), *Hirame novirhabdovirus* (HIRV), and *Snakehead novirhabdovirus* (SHRV) [1,2]. Novirhabdoviruses are enveloped and have negative-stranded RNA genome of approximately 11 kb [3–5] encoding five virion proteins (N, P, M, G, and L proteins) and a non-virion (NV) protein, whose gene is located between G and L genes. Novirhabdoviruses importantly affect both wild and farmed fish. For example, VHSV was isolated from more than 50 fish species from North America, Asia, and Europe, including around 15 farmed [6] and free-living marine fish species [7]. VHSV is a notifiable disease to the World Organisation for Animal Health (Office International des Epizooties, OIE, Paris, France).

The NV gene is approximately 500 nucleotides in length, corresponding to 12- or 14-kDa proteins in IHNV or VHSV, respectively [8]. In spite of the NV gene presence in the four rhabdovirus species mentioned above, NV proteins showed relatively high homology intraspecies but widely divergent interspecies sequences [8,9]. Regarding the NV functionality among novirhabdoviruses, while the *in vitro* or *in vivo* SHRV replication was not affected by the absence of NV [10] in warm-water flatfish, NV expression was required for a higher efficient replication of IHNV [11,12] or VHSV

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[13] in cold water rainbow trout or olive flounder, respectively. Comparative studies between wild-type (wt) and NV knockout IHNV or VHSV suggested that the NV protein may downregulate host *ifn1/mx* transcriptional levels during *in vitro* infection of rainbow trout gonad cells (RTG-2) [14] or cyprinid cells (EPC) [13], respectively. Furthermore, interferons (IFN)-induced *mx* transcript levels were higher in NV knockout VHSV than in wt-VHSV-injected flounder, suggesting that NV also interferes *in vivo* with IFN defences to favour VHSV replication [13]. It has also been put forward that VHSV suppresses TNF-α-mediated NF-κB activation through NV *in vitro* [15] and that NV from IHNV plays an anti-apoptotic function as detected by downregulation of caspase 3, 8, and 9 activities [16]. In a previous work using recombinant NV protein (rNV) from VHSV and a trout immune-targeted microarray, we confirmed not only previous results regarding *mx* and interferons downregulation and the anti-apoptotic role of NV but also a plethora of new gene expression changes (mainly downregulated) in immune innate and adaptive response genes (i.e.: tumour necrosis factors, antigen presentation, interleukins, complement) were described [17]. A previous work defined a nuclear location signal (NLS) sequence within the region located between the first and second α-helices in the IHNV NV (PRT strain) protein, with this NLS sequence being important for driving NV to the nucleus leading to a suitable inhibition of interferon-based responses and for obtaining normal replication levels [14]. This region could have different functional characteristics in VHSV NV due to the low homology between NVs from IHNV and VHSV. In the present work, we aimed to study the contribution of this particular region and other domains to NV function and subcellular location by using NV mutants and fragments. We assessed their ability to modulate the relative transcriptional expression of antiviral and immune response genes (*mx* and *il8*) in zebrafish (ZF4) and cyprinid-derived (EPC) cell lines. This work will provide better knowledge of novirhabdoviral NV proteins and could open up possibilities for future studies on molecular ligands as immune response targets.

2. Materials and methods

2.1. Cells and virus

The zebrafish (*Danio rerio*) ZF4 (ATCC© CRL-2050™) and EPC (*Epithelioma papulosum cyprini*, ATCC© CRL-2872™) cell lines were grown at 28 °C in a 5% carbon dioxide (CO_2) atmosphere in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, Thermo Fisher Scientific) for ZF4 and RPMI 1640 (Sigma-Aldrich, Spain) for EPC, both supplemented with 10% foetal bovine serum (PAA, Labclinics, Spain), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate (Gibco, Thermo Fisher Scientific) and Penicillin/Streptomycin 100 U/mL (Gibco, Thermo Fisher Scientific). For NV protein expression studies, the French VHSV 07.71 strain was used.

2.2. Plasmids expressing NV, NV mutants, and NV fragments

The DNA sequence corresponding to the VHSV NV 07.71 strain (GenBank nº AJ233396) isolated from rainbow trout [18] was used in this study. In order to establish and delimit the functional domains of the NV 07.71 molecule, the DNA sequence was split into different fragments for their expression and evaluation of their inducing effects. So, we constructed eight fragments (F1 to F8) covering secondary structure domains (Fig. 1) based on the SSpro package prediction model [19] (http://scratch.proteomics.ics.uci.edu). On the other hand, eleven different NV 07.71 mutants were obtained by changing the respective amino acid to alanine at positions D28A, N31A, L33A, N34A, C35A, D36A, F37A, D38A, R39A, S40A, and D41A (Fig. 2). Three of them were constructed in the lab (L33A, D36A, and R39A) while the others were obtained by chemical synthesis (Invitrogen, Thermo Fisher Scientific, USA) and then subcloned in the lab. In addition, and in order to compare the NV expression effects upon transfection among novirhabdoviruses, we also constructed plasmids for IHNV NV (strain Oregon69, UniProtKB Q08455), HIRV NV (GenBank U47847.1), and SHRV NV (GenBank NC_000903.1). Moreover, for NV location assays we used some of the plasmids described above and also constructed the plasmids expressing IHNV NV (PRT strain) as well as the new hybrid VHSV NV 07.71 with the nuclear location sequence (NLS) EGDL (NV-EGDL) inserted between residues LNCDF and DRSD after amino acid 37 (Supplementary Fig. S6A), with all the proteins bearing a T7 tag at 3′-end. Only IHNV NV PRT strain was cloned into pCDNA expression plasmid as described by Choi et al. [14]. Otherwise specified, all the coding sequences were inserted into the pMCV1.4 mammalian expression plasmid [20].

2.3. Predicted 3D models

Three-dimensional protein models were predicted using 3Dpro (http://scratch.proteomics.ics.uci.edu) since this method worked with the best accuracy for caspase 6 x-ray crystallographic structures (http://scratch.proteomics.ics.uci.edu/casp6_results.html). For each NV model, the 3Dpro package set up a PDB file that was graphically represented with the UCSF Chimera software [21]. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics (University of California, San Francisco).

2.4. Cell transfection assays

With a view to expressing the different proteins, 1.9×10^5 cells seeded in 24-well plates were transfected using Lipofectamine® 3000 reagent (L3000-008, Invitrogen, Thermo Fischer Scientific) according to the manufacturer's instructions. Transfected cells were incubated at 28 °C for 24 h and further processed for immunofluorescence assays (see below) or used for transcriptomic studies. In the latter case, cells at 24 h post transfection (hpt) were treated with poly I:C (final concentration 25 μg/mL) for 20 h in order to induce the activation of the cells. Finally, the cells were harvested and the total RNA was extracted and cDNA synthesis was carried out as indicated below.

2.5. Quantitative real-time PCR (qPCR)

Total RNA was isolated from ZF4 cells using E.Z.N.A HP Total RNA Kit (R6812-01 Ω Bio-Tek) and was then eluted in 40 μL of RNase-free water. RNA concentrations were measured by Nanodrop 1000 (Nanodrop, Thermo Fischer Scientific). Total RNA was then reverse transcribed using oligo-dT and PrimeScript™ reverse transcriptase (RR037A TAKARA, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (RR420A TAKARA, Japan) as well as the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). Reactions were carried out in a 20 μL reaction volume containing 10 ng of cDNA. Zebrafish transcripts of the orthomyxovirus resistance gene (*mxa* and *mxb* isoforms), interleukin 8 (*il8*) and ribosomal protein large P0 (*rplp0*) genes were determined through qPCR with specific primers (see Supplementary Table 1). For each experiment, the expression level of the analysed genes was calculated using the $2^{-\Delta Ct}$ relative quantitation method. The Ct for each gene was normalised to the corresponding *rplp0* Ct value by applying the formula $\Delta Ct = Ct_{gene} - Ct_{rplp0}$. The gene *rplp0* was chosen as an internal reference because it has been widely used for normalisation of the expression levels after stimulation with or

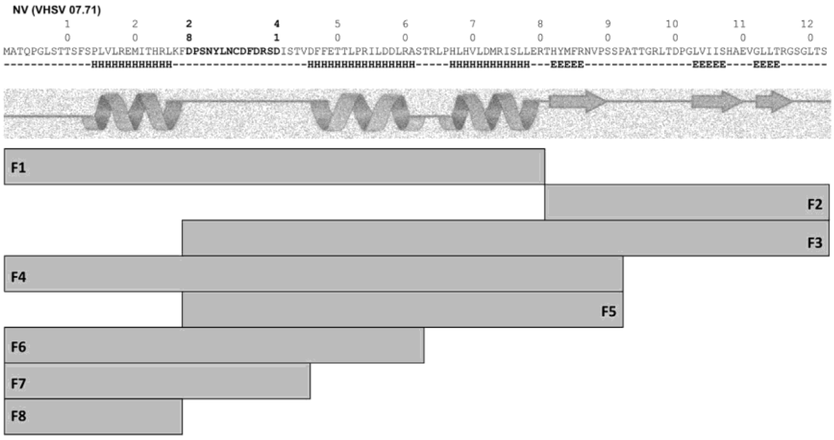

Fig. 1. VHSV NV 07.71 was split into different fragments (F1 to F8) based on predicted secondary structure with the SSpro 5.2 program (http://scratch.proteomics.ics.uci.edu) so as to establish the functional domains. These fragments were cloned into pMCV1.4 vector and transfected in ZF4 cells. The *mx* and *il8* induced transcript levels were measured. For immunofluorescence assays, another set of constructs was obtained by adding GFP at 3′-end of each fragment.

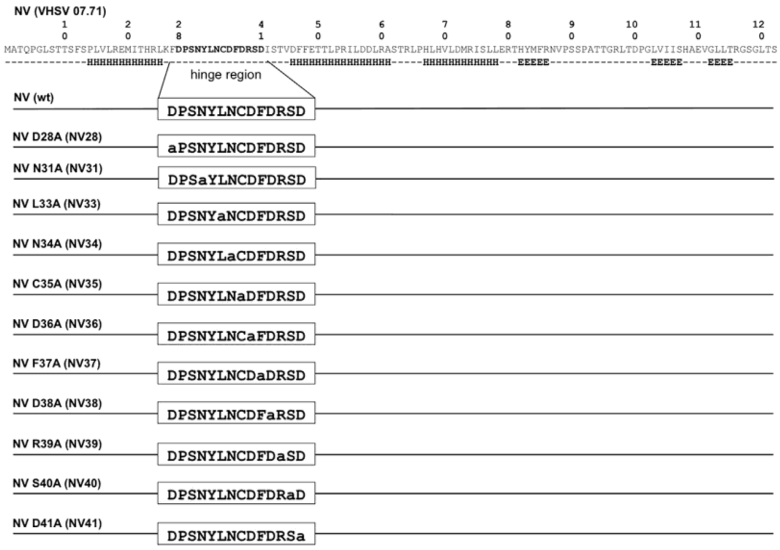

Fig. 2. Schematic diagram of the VHSV NV 07.71 amino acid sequence depicting the eleven mutants generated by alanine substitution at positions 28, 31, and from 33 to 41. Another set of plasmids was constructed by adding GFP at the 3′-end of each of the constructions. For different assays, the pMCV1.4-GFP plasmid was used.

without poly I:C [22–25]. In addition, our data showed that *rplp0* threshold (Ct) values were very similar among transfected cells with the different NV constructions and further stimulation with poly I:C, thus discarding any variation of *rplp0* induced by poly I:C. The specificities of each primer pair were confirmed by melting curve analysis (and agarose-gel electrophoresis).

2.6. Nuclear and cytoplasmic analysis of NV by western blot

EPC cells on P24 dishes (1.5×10^5 cells) were infected with a multiplicity of infection (MOI) of 0.5 with VHSV 07.71 for 24 h. Then, cells were harvested and cytoplasmic and nuclear protein extracts were obtained using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific™, USA) following the manufacturer's instructions. Both cytoplasmic (10% of total protein) and nuclear protein (30% of the total) extracts were submitted to SDS-PAGE, transferred onto Hybond ECL membranes (GE Healthcare Europe GmbH, Spain). Subsequently, the membrane was incubated with a rabbit polyclonal anti-NV (1/400) for 1 h. This polyclonal antibody was obtained against RILDDLRASTRLPH peptide from NV 07.71 (GenScript Inc, USA). After washing with PBS-Tween 20 (0.1%, PBST), the membrane was incubated with goat anti-rabbit horseradish peroxidase (1/1500) for 30 min and then washed with PBST. Immunoreactivity was detected using home-made ECL reagents and photographed with the Gel DocTM XR + system (BioRad, Spain).

2.7. Immunofluorescence and confocal microscopy

ZF4 or EPC cells were seeded and grown on glass coverslips in P24 plates (SPL Life Sciences, Korea). Transfected cells with various types of plasmids were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT), washed with PBS and further treated with ice-cold methanol for 10 min at 4 °C, and finally washed with PBS. Those constructions fused to GFP could be directly observed by fluorescence microscopy. We also conducted assays for the detection of VHSV NV (wild-type and mutants) without tags, whereas other transfections were carried out with VHSV NV-EGDL, IHNV NV (PRT and Oregon69), and HIRV NV constructions with a T7 Tag at 3′-end. In all assays, cells were blocked with buffer (BSA 2%, Triton 3% in PBS) for 60 min after fixing and permeabilisation. For untagged VHSV NV, the rabbit polyclonal antibody described above was used at 1/200 for 60 min. For T7-tagged proteins a rabbit polyclonal anti-T7 antibody (AB3790, Merck Spain) was used at 1/400. After washing, cells were incubated with goat anti-rabbit A488 at 1/800 (Molecular Probes™, Thermo Fischer Scientific, Spain). Finally, nuclei were stained with TO-PRO®-3 (Molecular Probes™, Thermo Fisher Scientific, Spain) in PBS at a 1/1000 ratio. After washing, coverslips were finally mounted on glass plates. All cells were observed under a Leica TCS SP2-AOBS confocal microscope (Leica-Microsystems, Wetzlar, Germany) using a 63 × immersion oil objective.

2.8. Statistical analysis

Statistical differences were analysed using the one-way ANOVA and the Student's t-test (GraphPad Prism v6), and were considered to be significant at $p \leq 0.05$. Unless otherwise stated, results are expressed as arithmetic means ± standard error (SE). All experiments were repeated at least three times as independent assays.

3. Results

3.1. VHSV NV variability intraspecies and structural NV similarities interspecies

The numerous available protein sequences from VHSV NV allowed their alignment and comparison, which showed a high degree of variability with a 37.7% of identity and conserved positions are located throughout the protein (supplementary Fig. S1). Therefore, the delimitation of the region which could be implicated in function was not obvious. Moreover, there are a few shared amino acids between VHSV NV (07.01) and IHNV NV (Oregon69) (supplementary Fig. S2). Despite dissimilar primary sequences, the predicted secondary structure (SSpro 5.2 program, http://scratch.proteomics.ics.uci.edu) of VHSV NV, IHNV NV, and HIRV NV depicted similar arrangements of α-helix, especially at the beginning of the molecules (supplementary Fig. S3), thus suggesting structural similarities among them.

3.2. Splitting the VHSV NV: N-terminal part of the molecule induces low mx and il8 levels

The NV gene was split into eight fragments (F1 to F8, Fig. 1), so that we could study the functional ability of each NV fragment to inhibit the *mx* and *il8* gene expressions and to compare them with those of the complete VHSV NV 07.71 protein. Because NV is able to inhibit the *mx* upregulation driven by poly I:C, ZF4 transfected cells were treated with poly I:C so that the real inhibitory capacity of each NV fragment could be assessed. Our transfection experiments showed an efficiency of 19.7% ± 4.5% of the standard error of the mean (SEM). Although the transfection efficiencies of fish cells tend to be low, the percentage of transfected cells may be useful for the interpretation of results.

The results showed that only F2 clearly upregulated *mx* and *il8* transcript levels when compared to NV, while the other fragments induced similar *mx* and *il8* expression levels to NV, despite a slight *mx* increase registered in F5 and F8 (Fig. 3). The fragment F1 has a secondary structure mainly composed of α-helices and a coil segment (termed hinge region) between the two first α-helices. The fragments F3 to F8 also contain at least one of the two first α-helices and/or the hinge region (Fig. 1). These structures could be enough for maintaining a similar activity to the complete NV. On the contrary, the fragment F2 has a different secondary structure, which could explain why F2 did not retain the inhibition capacity of the complete NV.

3.3. Mutated amino acids that improve NV function

In order to study the contribution of amino acids from the hinge region to *mx*- and *il8*-induced levels, we studied NV-derived mutants at positions 28, 31, 33, 34, 35, 36, 37, 38, 39, 40, and 41, changing each corresponding amino acid to alanine (Fig. 2). The hinge region was demonstrated to be very important for IHNV NV location and function. These amino acid positions were chosen due to: 1) conserved amino acid residues at positions 28, 31, 33, 34, and 36 (Table 1); 2) shared amino acids between VHSV NV 07.01 and IHNV NV PRT (positions 33, 37, and 41); and 3) other amino acids surrounding the mentioned positions (Fig. 1 and supplementary Figs. S1 and S3). The capacity of NV mutants for modulating *mx* and *il8* gene expressions in transfected ZF4 was evaluated and compared with that of wt VHSV NV 07.71. Results showed that mutants NV34, NV35, NV38, and NV40 upregulated ZF4 *mx* transcript levels (Fig. 4A) while mutants NV28, NV31, NV33, and NV37 induced similar *mx* levels to wt-NV. Surprisingly, mutants NV36 (D36A), NV39 (R39A), and NV41 (D41A) induced a stronger *mx* downregulation than wild-type NV (Fig. 4A) that was statistically significant ($p \leq 0.05$). The *il8* transcript levels were similar among almost all the mutants and wt-NV (Fig. 4B), and there was no correlation with those mutants which upregulated *mx*.

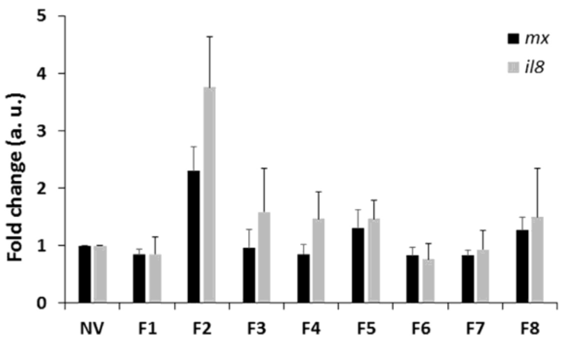

Fig. 3. Fold change of the *mx* (black bars) and *il8* (grey bars) transcript levels (arbitrary units, a. u.) obtained from ZF4 cells transfected with constructions expressing the different NV fragments (F1 to F8) and the complete wt-NV (NV 07.71) as a control. After 24 hpt, ZF4 cells were treated with poly I:C (25 μg/mL) for 20 h. Then, the cells were collected and the total RNA was retrotranscribed to cDNA and analysed through qPCR. Transcription levels were calculated by applying the formula: potency = $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{gene} - Ct_{rplp0}$ and by using *rplp0* gene as housekeeping. Fold change was achieved for *mx* by dividing each *mx* value/*mx* wt-NV. Fold change calculations for *il8* were identical. The results are represented as the means ± standard error of mean (SEM). At least three independent assays were carried out.

Notwithstanding, again mutants NV36 (D36A), NV39 (R39A), and NV41 (D41A) induced a stronger *il8* downregulation than wild-type NV that was statistically significant ($p \leq 0.05$) (Fig. 4B). These results indicate that the NV mutants at positions 36, 39, and 41 may allow a better stabilization (first and second α-helices) of the molecule and an improved interaction with ligand(s). On the other hand, there is no clear correlation between conserved positions (Table 1) and the gain or loss of function, except for positions 36 and 39, which exhibited a strong effect (downregulation) on both *mx* and *il8* transcripts. In addition, we also compared the *mx* downregulation ability of NV from different novirhabdovirus (Fig. 4A). NV from Snakehead novirhabdovirus (SHRV) did not downregulate *mx* and this fact would confirm that this NV is not involved in pathogenesis [10]. On the other hand, IHNV NV and HIRV NV had the capacity to downregulate *mx*. Interestingly, VHSV NV41 mutant seems to have the most powerful capacity for *mx* downregulation among all the NV novirhabdoviral molecules studied.

A 3D model prediction of the VHSV NV 07.71 protein obtained using 3Dpro (Baldi group server, http://scratch.proteomics.ics.uci.edu) showed the spatial orientation of the residues 36, 39, and 41 (Fig. 5A). The predicted structure showed that residues D36 and R39 might have surface contact with each other (Fig. 5A), thus giving rise to a certain stabilization and compensation of the charges in wild-type NV. In addition, the D41 lateral chain seems not to be interacting with other residues of NV as it is directed towards the outside (Fig. 5B). The 3D structures were also predicted for mutants taking into account the changes D36A (Fig. 5B), R39A (Fig. 5C), and D41A (Fig. 5D). These predicted models presented structures which were very dissimilar to each other and to that of wt-NV. Restricted to the hinge region, the 3D model for mutant D41A showed similar interaction to wt-NV between D36 and R39 amino acids (Fig. 5A and D). D36A, R39A, and D41A lead to overall conformational structure changes of the NV protein which enhance its inhibitory capacity. Predicted 3D models did not help to clarify the correlation between mutated D36A, R39A, and D41A and their improved inhibitory function.

3.4. *VHSV NV, derived mutants and F1 fragment have a cytosolic location*

In order to study the location of the VHSV NV protein, we used immunofluorescence (IF) to observe the expression of transfected ZF4 or EPC cells with plasmids coding for wt-NV, NV mutants (L33A, N34A, C35A, D36A, F37A, D38A, R39A, S40A, and D41A) and NV fragments (F1, F2, F6, F7, and F8), tagged with GFP at their 3′-end. Moreover, wt-NV and mutants without GFP were also studied through IF by using rabbit polyclonal antisera against VHSV NV (see above). In addition, to compare the cellular location among the novirhabdoviral NV proteins, the IHNV NV (Oregon69) and HIRV NV were also transfected and detected with an anti-T7 polyclonal antibody as primary antibody and Alexa-488 as secondary antibody. Both wild-type NV and NV mutants with fused GFP (Fig. 6) or without GFP (supplementary Fig. S4) showed a cytoplasmic location pattern, sometimes with certain perinuclear accumulation. On the contrary, T7-tag expressed IHNV NV and HIRV NV proteins were located at nucleus (Fig. 6C and D). In order to confirm the subcellular location of VHSV NV 07.71, nuclear and cytoplasmic fractions of EPC cells infected with VHSV 07.71 were analysed using Western blot and a rabbit anti-NV 07.71 antibody (Fig. 7). When compared to purified VHSV NV (lane 3), the cytoplasmic fraction (lane 2) was positive whereas the nuclear fraction (lane 1) was negative for the presence of NV 07.71.

On the other hand, when NV fragments fused to GFP were analysed at 24 hpt, the subcellular distribution was similar among them, being both nuclear and cytoplasmic (i. e.: F2GFP, F6GFP, and F8GFP) except for F1GFP, which had a cytoplasmic distribution like the whole NV protein (Fig. 8). The only difference between F1 and F6 is the absence of the third α-helix in F6, and this sequence seems to be important for cytoplasmic location. Fragment F1 induced similar *mx* and *il8* transcript levels to wt-NV and also shared the same location.

4. Discussion and conclusion

Non-virion (NV) proteins from different novirhabdoviruses are dispensable but necessary for an efficient virus replication, except for SHRV. These proteins are important for virus replication by inhibiting the interferon system, apoptosis and several innate and adaptive immune-related genes. Despite dissimilar primary NV protein sequences among VHSV, IHNV and HIRV, these proteins produce analogous inhibitory effects. It is unknown whether this might be related to linear polypeptide sequences or due to a more complex tertiary protein structure, which is not shared by SHRV NV (Supplementary Fig. S3). In the present work, we have determined the contribution of different NV regions to the modulation of *mx* and *il8* transcript levels and their subcellular location. Moreover, we have also established the contribution of individual amino acids from the region between first and second α-helices (hinge region) to the VHSV NV function. The N-terminal part of NV (i.e.: fragment F1) induced a similar *mx* and *il8* modulation to wt-NV and also shared the same cellular location. However, fragment F6 induced similar *mx* and *il8* levels to those of F1 and wt-NV but did not share the same location pattern. This data suggests that the third α-helix absent in F6 could be important for a better tertiary structure and correct location. The NV function has been related to its location and interaction with mitochondria [26]. More studies are needed to confirm that F1 but not F6 could interact with mitochondria. On the other hand, the C-terminal part of NV (i.e.: fragment F2) plays an unknown role in the cell; it upregulated *mx* and *il8* levels and did not have the characteristic cytoplasmic location pattern of the NV

Table 1
Amino acid variability and geographic distribution among VHSV NV proteins from position 28 to 41 within the hinge region.

NV Isotype[a]	Amino acid position within the hinge region														Geographic distribution
	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
1[b]	D	P	S	N	Y	L	N	C	D	F	D	R	S	D	Atlantic [9,28]
2	D	P	S	N	Y	L	N	C	D	L	D	R	S	D	Atlantic [8,29,30]
3	D	P	S	N	Y	L	N	C	D	I	D	R	S	D	Atlantic [31,32]
4	D	P	S	N	Y	L	N	C	D	F	N	R	S	D	Atlantic [9,32]
5	D	P	S	N	Y	L	N	S	D	L	D	R	S	E	Asiatic [33,34]
6	D	P	S	N	Y	L	N	C	D	I	D	R	S	E	Great Lakes [35]

[a] NV isotypes are defined attending to the hinge region. Studied amino acid positions are underlined.
[b] NV 07.71 belongs to isotype 1. Isotypes 1, 2, 3 and 4 were found in Atlantic Ocean species; isotype 5 was found in Korean, Chinese and Japanese species; and isotype 6 was found in Laurentian Great Lakes.

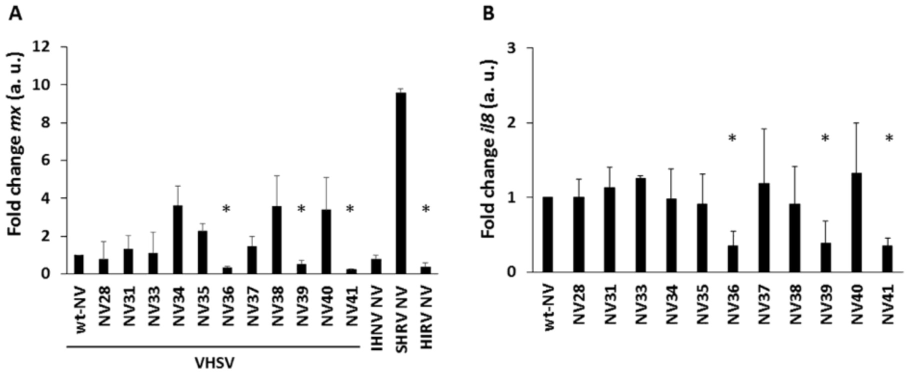

Fig. 4. Fold change of the *mx* (A) and *il8* (B) expression levels (arbitrary units, a. u.) obtained from ZF4 cells transfected with constructions expressing the different NV proteins: wild-type (wt) NV (VHSV NV 07.71), VHSV NV 07.71 mutants (NV28, NV31, and NV33 to NV41), NV from IHNV (Oregon69), NV from HIRV, and NV from SHRV. After 24 hpt, ZF4 were treated with poly I:C (25 μg/mL) for 20 h. The cells were then collected and the total RNA was retrotranscribed to cDNA and analysed by qPCR. Transcription levels were calculated by applying the formula: potency = $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{gene} - Ct_{rplp0}$ and by using *rplp0* gene as housekeeping. Fold change was achieved for *mx* by dividing each *mx* value/*mx* wt-NV. Similar calculations were carried out for *il8* normalisation. The results are represented as the means ± standard error of mean (SEM). At least three independent assays were carried out. Asterisks denote statistically significant differences.

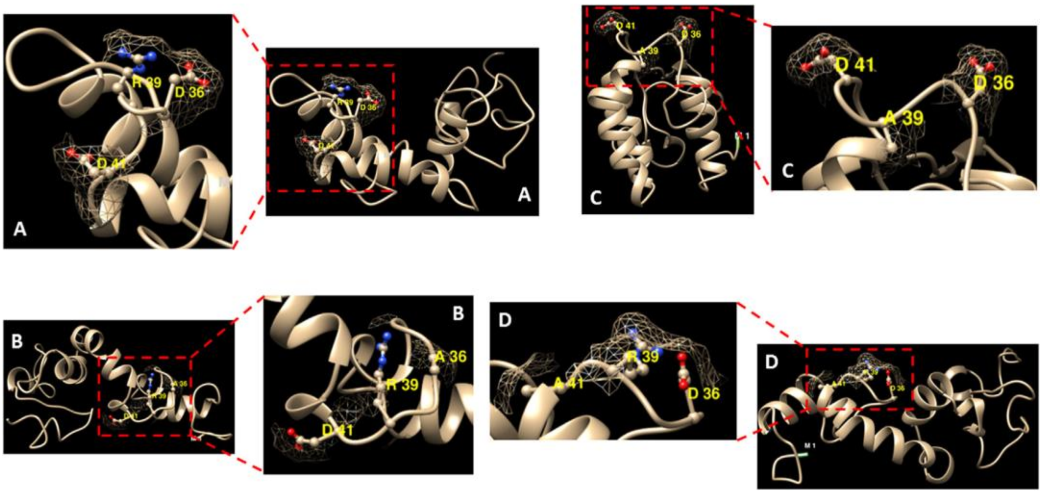

Fig. 5. Three-dimensional modelling of the wild-type VHSV NV 07.71 protein (A) and mutants NV D36A (B), NV R39A (C), and NV D41A (D) obtained using 3Dpro (Baldi group server, http://scratch.proteomics.ics.uci.edu). The figure shows the complete predicted structure and a close view of these amino acids. Alanine substitution of the amino acids D36, R39 or D41 improved the function of the NV mutant with respect to wt-NV.

protein and its F1 fragment. We have also determined that VHSV NV 07.71 and all mutants studied have a cytoplasmic location and share this characteristic with VHSV NV Fil3 strain [27]. There are amino acid differences between NVs from 07.71 and Fil3 strains (Supplementary Fig. S5) but they do not affect the cellular location. Probably, all VHSV NV proteins share the same characteristic of the cytoplasmic location due to the lack of a canonical or non canonical NLS (nuclear location sequence). The subcellular location is different for IHNV NV because an NLS (EGDL motif) has been described for IHNV NV (PRT strain) [14]. We have determined that IHNV NV (PRT and Oregon69 strains) and HIRV NV are located at nucleus (Fig. 6C, D and S6B), and the responsible for this location is an NLS which is different among those NV proteins: KEGDL motif for IHNV NV PRT, DDGDL motif for IHNV NV Oregon69, and DDGKI for HIRV NV. This NLS motif is also situated in the homologous region between the two first α-helices of the IHNV NV (hinge region) and it has been correlated with IHNV pathogenesis [14]. Therefore, despite the low homology of the hinge region between IHNV and VHSV NVs (see results and supplementary Fig. S2), this region is important for function at distinct cellular locations. As an approach, we carried out the insertion of the IHNV NV nuclear location signal from PRT strain (EGDL motif) into the hinge region of the VHSV NV 07.71 between residues LNCDF and DRSD after amino acid 37 for obtaining NV-EGDL (supplementary Fig. S6A). However, this modification was not enough to redirect NV-EGDL to the nucleus, thus, remaining at cytoplasm (supplementary Fig. S6C), unlike IHNV NV PRT (supplementary Fig. S6B). Very recently, it has been described that both NV from VHSV and IHNV interact with protein phosphatase, 1Bb (PPM1Bb), which is a member of the PPC2 family and which dephosphorylates TBK-1 [26]. Nuclear NV (IHNV, HIRV) and cytosolic NV (VHSV) proteins seem to interact with PPM1Bb in a different manner; IHNV NV could bind to PPM1Bb mainly at the nucleus, acting on substrates different from TBK1 [26]. VHSV NV recruits PPM1Bb around mitochondria to dephosphorylate TBK1, and further interferes with the retinoic acid-inducible gene-I (RIG-I) response. Our data suggested that F1, and

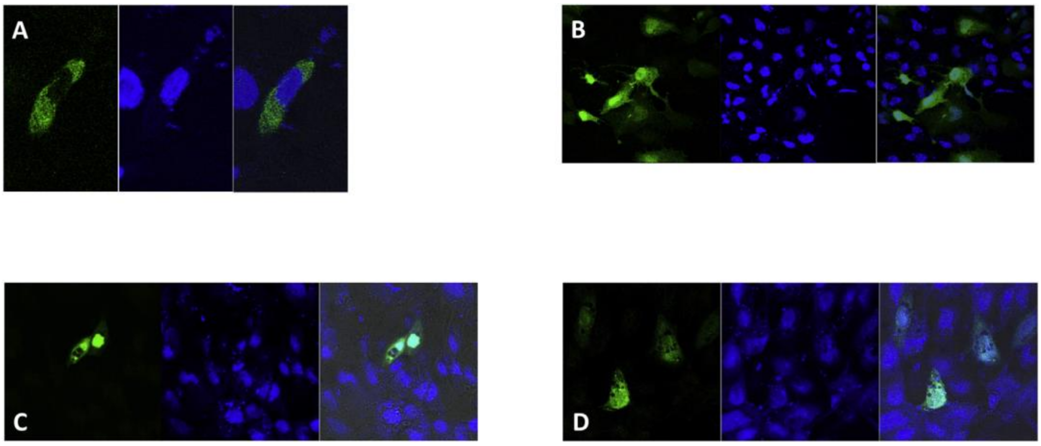

Fig. 6. EPC cells were transiently transfected for 24 h with all pMCV1.4 plasmids expressing VHSV NV (wt and mutants) with a GFP Tag at their 3′-end and with the control pMCV1.4-GFP. For the complete study of the cellular location pattern of the distinct NV proteins, the plasmids expressing IHNV NV (Oregon69) and HIRV NV were also transfected and detected with an anti-T7 polyclonal antibody as primary antibody and Alexa-488 as secondary antibody. Immunofluorescences were examined under confocal microscopy. The representative panels show results for VHSV NV39-GFP (A), the GFP as control (B), IHNV NV (C) and HIRV NV (D). VHSV NV mutants were clearly located in the cytoplasm as wild-type NV 07.71 (not shown), whereas IHNV NV and HIRV NV were located at nucleus. The left panels show GFP-tagged NV (A, B) and T7-tagged NV (C, D) staining, the right panels show a merge with nuclei staining (TO-PRO®-3.). Each image is representative of the majority of the cells observed in several fields.

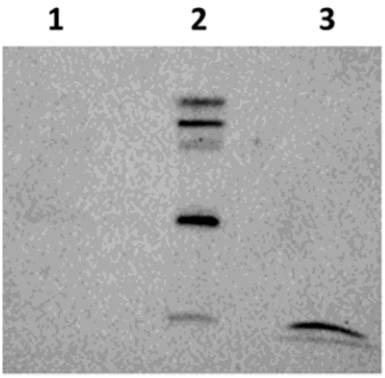

Fig. 7. Western-blot of the cytoplasmic and nuclear fractions from EPC cells (1.5×10^5) infected for 24 h with VHSV NV 07.71 at MOI = 0.5. Cytoplasmic and nuclear fractions were prepared and submitted to SDS-PAGE to detect NV with an anti-rabbit polyclonal antibody. NV was detected at cytoplasmic fraction (lane 2) when compared to purified NV as control (lane 3), but could not be detected in the nuclear fraction (lane 1).

not shorter N-term fragments nor F2, maintained a proper cytosolic location for a possible and suitable interaction with PPM1Bb. This would need to be confirmed in future experiments. On the other hand, results demonstrated that mutants D36A, R39A, and D41A increased *mx* and *il8* downregulation and could suggest a better stabilization of the NV molecule and an improved interaction with PPM1Bb for inducing a stronger TBK1 dephosphorylation. This would also lead to lower *mx* and *il8* expression transcript levels. Further research on different VHSV NV proteins is needed in order to confirm whether the same mutations at positions 36, 39, and 41 would lead to an improved inhibition similar to that observed in this study with VHSV NV 07.71. In addition, future studies would help to clarify the mechanisms implied in the interaction of the wt-NV and mutants with PPM1Bb. Among the available VHSV NV amino acid sequences, there is no alanine at neither of the studied residues (Table 1) and residues at positions 36 and 39 are conserved whereas amino acids are variable at position 41 (D or E). It is interesting to note that a mutation incidentally conducting alanine at positions 36, 39 or 41 in the NV protein might produce a more

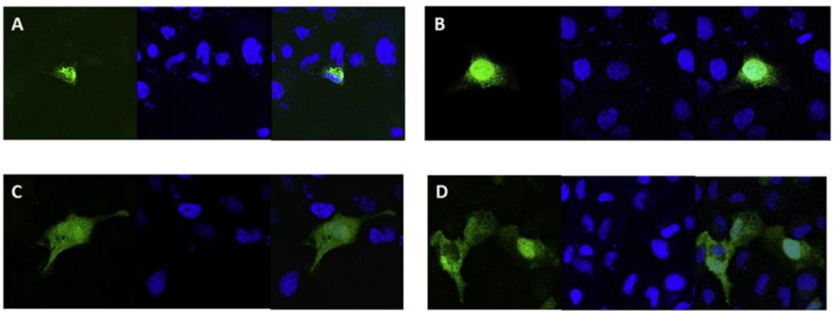

Fig. 8. EPC cells were transiently transfected for 24 h with pMCV1.4 plasmids expressing fragments fused to GFP at 3′-end. Immunofluorescences were examined under confocal microscopy. While the location of F1GFP is only cytoplasmic (dotted pattern) (A), the expressed F2GFP (B), F6GFP (C) and F8GFP (D) are located at both nucleus and cytoplasm. The location of F1GFP was also confirmed in transfected CHSE-214 cells. The fragment F1, and not F2, maintained an identical location of wt-NV. Each image is representative of the majority of the cells observed in several fields. Nuclei (blue) were stained with TO-PRO®-3.

pathogenic VHSV strain. Despite the different primary protein sequences among IHNV and VHSV NVs, here we demonstrated that the VHSV NV homologous region (hinge region) is also important for function. The results presented in this work can help to better understand the host shut-down mechanisms driven by non-virion proteins and to define the importance of VHSV NV regions and residues for function. This study may also constitute the basis for future interaction studies with PPM1Bb or other complexes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2017.09.021.

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PAPER III: *Optimization of neutralizing antibody assays*

OPTIMIZATION OF FIXED-PERMEABILIZED CELL MONOLAYERS FOR HIGH THROUGHPUT MICRO-NEUTRALIZING ANTIBODY ASSAYS: APPLICATION TO THE ZEBRAFISH/VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) MODEL

La respuesta inmune adaptativa, basada principalmente en el desarrollo de anticuerpos neutralizantes, conlleva la supervivencia de los peces tras la infección por VHSV. La determinación de estos anticuerpos neutralizantes se realiza normalmente con volúmenes grandes de suero/plasma y mediante el contaje de unidades formadoras de placa (pfu) después de una incubación de células en monocapa con el virus. Alternativamente, se pueden reducir los tiempos de incubación, hacer una inmunodetección y contar los focos formados (ffu). No obstante, en truchas supervivientes a la infección por VHSV sólo se han detectado anticuerpos neutralizantes en el 40-50% de los peces estudiados y mediante ensayos de ffu. Además, las técnicas utilizadas hasta ahora requieren de pasos de lavado exhaustivos que conllevan la pérdida de células, un contaje de focos (ffu) aproximado y, en definitiva, una menor sensibilidad. En este trabajo se establece un nuevo método de seroneutralización para determinar la presencia de anticuerpos neutralizantes (IgM) de pez cebra (*Danio rerio*) en los peces supervivientes a los 2 meses post-infección con VHSV. El método combina el uso de volúmenes muy pequeños de plasma con una fijación-permeabilización específica (PERMFIX), y una detección por citometría de flujo. Para ello, se usaron células EPC dispensadas en placas de poly-D-lisina, que se infectaron con una mezcla pre-incubada de plasma de pez cebra con VHSV. Tras la infección de la monocapa, las células se fijaron con formaldehído para disminuir su pérdida en los lavados. Después, se permeabilizaron las células con saponina, se incubaron con un anticuerpo monoclonal anti-N de VHSV, se levantaron las células con tripsina y se analizaron por citometría de flujo. Los resultados mostraron una correlación de r=0.99 entre el método ffu (tinción con DAB) y este nuevo método, y usando entre 200 ffu y 500 ffu de VHSV por 50000 células EPC. Este método semi-automático, rápido y práctico permitió detectar anticuerpos neutralizantes anti-VHSV en 3 μL de plasma en el 95.1% de los peces cebra supervivientes a la infección por VHSV. Así, esta nueva metodología podría analizar el suero/plasma de peces de diversos tamaños en condiciones estándar y determinar la presencia de anticuerpos neutralizantes anti-VHSV en los mismos, pudiendo ser aplicada a otros modelos virales.

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Optimization of fixed-permeabilized cell monolayers for high throughput micro-neutralizing antibody assays: Application to the zebrafish/viral hemorrhagic septicemia virus (vhsv) model

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ABSTRACT

A new high throughput centrifugation-free method to estimate viral neutralizing antibody levels in low volumes and large numbers of plasma blood samples is described. Cell monolayers were, (i) plated on poly-D-Lys coated 96-wells, (ii) infected with viruses previously incubated with fish plasma containing antibodies, (iii) fixed with formaldehyde to increase cell recovery and avoid centrifugation steps, (iv) permeabilized with Saponin, (v) immunostained in the presence of Saponin by using a monoclonal antibody (MAb) to viral protein, (vi) digested with trypsin to detach cells from the monolayer, in the absence of Saponin to reduce damage of intracellular MAb-antigen complexes, and (vii) gated by flow cytometry using automatic 96-well batch analysis. The method was applied to the determination of plasma neutralizing antibodies from zebrafish (*Danio rerio*) surviving infections with viral hemorrhagic septicemia virus (VHSV) (an important rhabdovirus of salmonids). This semi-automatic, rapid and practical assay detected anti-VHSV neutralizing antibodies in the plasma (~3 μl per fish) of 95.1% of the zebrafish surviving VHSV infections. The fixed-permeabilized monolayer (FIXPERM) micro-neutralization method might help to analyze sera/plasma from small fish under standarized high throughput conditions.

1. Introduction

There are many methods described to estimate *in vitro* anti-viral neutralizing antibody levels by cell cultures. Most of them rely on counting visible plaques of cell lysis (plaque forming units, pfu) after 3–7 days incubation of infected cell monolayers. Overlaid with semisolid media (*i.e.* agarose, methylcellulose, *etc.*) is used to reduce spreading of viral progeny (Hosie et al., 2011; Klasse and Sattentau, 2002; Rainwater-Lovett et al., 2012). Alternatively, viral spreading can be reduced by shorter incubation times, immunostaining and counting focus (focus forming units, ffu) with the aid of an inverted microscope (Chinchilla et al., 2013; Lorenzo et al., 1996). However, those methods counting pfu require larger volumes of sera/plasma than ffu and therefore are not suitable for small fish. Because both pfu and ffu assays require visual counting, theoretically they could be improved by automation of the determination of the numbers of infected cells by flow cytometry.

While a few reports do exist on the use of flow cytometry as an alternative to plaque/focus viral neutralization assays, this method has not been widely used (Kraus et al., 2007; Sashihara et al., 2009). Among other things, the many centrifugation steps required for washing during immunostaining reduce cell recovery and/or increase damage of infected cells. In addition, low sensitivity due to high backgrounds when immunostaining intracellular viral proteins might be a problem depending on the cells, viruses and/or anti-viral antibodies used with each particular assay. On the other hand, to avoid centrifugations some authors have reported methods for cell monolayer fixation and final trypsin digestion to suspend cells before flow cytometry (Grabner et al., 2000). Other authors reported that the best intracellular detection of viral antigens by flow cytometry was obtained by using reversible permeabilization of cellular membranes with Saponin (Gerner et al., 2008). By combining the above two mentioned methodologies (Gerner et al., 2008; Grabner et al., 2000) with fixation to poly-D-Lys coated wells, a high throughput centrifugation-free assay has been developed based on a ffu micro-neutralization method described before (Chinchilla et al., 2013; Lorenzo et al., 1996). The new assay has been applied to zebrafish (*Danio rerio*) and viral hemorrhagic septicemia virus (VHSV, an important rhabdovirus of salmonids) (Encinas et al., 2010; Novoa et al., 2006). The study was

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focused in zebrafish because despite the growing interest in this fish as a disease/immunological model, few studies have addressed their antibody response to viruses. The small amounts of blood that can be harvested from individual zebrafish, the limited access to reagents to detect their antibodies and the scarce number of methods described to estimate viral antibodies in large numbers of samples, increased the difficulties to study zebrafish antibody responses to viruses.

The presence of zebrafish immunoglobulin M (IgM)-dependent neutralizing antibodies in plasma from zebrafish surviving VHSV infections was demonstrated previously by using a micro-neutralization ffu assay (Chinchilla et al., 2013). Micro-neutralization reduced cell culture time and minimized both the use of zebrafish plasma and amounts of reagents. Thus, VHSV-infected ffu of 5–12 cells in 96-wells could be counted under an inverted microscope ~24 h after infection of the cell monolayers (Chinchilla et al., 2013). Although, due to the low amounts of blood this micro-neutralization assay by ffu was more practical to use in zebrafish than any other possible alternative pfu assays (for instance, those using agarose or methylcellulose), the analysis of large numbers of samples still required time-consuming labor-intensive visual counting. Flow cytometry was a possible alternative to speed up the infected cell determinations.

To obtain an maximal differentiation between non-infected and VHSV-infected cells by intracellular immunostaining of VHSV N nucleoprotein, poly-D-Lys plates, fixation and reversible permeabilization of fish monolayers (fixed-permeabilized monolayers, FIXPERM), had to be optimized. By using extracellular trypsin digestion in non-permeabilizing buffers, the immunostained cell monolayers could be then converted to cell suspensions for flow cytometry without altering antigen-antibody complexes. Combination of the FIXPERM method with flow cytometry using 96-well batch analysis allowed for a practical and rapid management of large number of samples (high throughput). Combination of all these methodologies, allowed the determination of anti-VHSV neutralizing antibody levels in low volumes and large numbers of plasma obtained from individual zebrafish surviving VHSV infections. Because zebrafish is susceptible to infections caused by other rhabdoviruses naturally infecting other fish species (LaPatra et al., 2000; Phelan et al., 2005; Sanders et al., 2003), the FIXPERM micro-neutralization assay could be used to follow up any of those rhabdoviral infections. Zebrafish antibodies against other viruses or in any other small fish can be estimated by fine tunning the FIXPERM micro-neutralization method to each case.

2. Material and methods

2.1. Virus and fish cell culture

The viral hemorrhagic septicemia virus (VHSV) strain 07.71 (VHSV-07.71) isolated in France from rainbow trout *Oncorhynchus mykiss* (LeBerre et al., 1977) was replicated in monolayers of EPC cells, from the fathead minnow fish (*Pimephales promelas*), obtained from the American Type cell Culture (ATCC) collection catalog CRL-2872 (Manassas, Virginia, USA). EPC cells were grown at 28 °C with an atmosphere of 5% CO_2 in RPMI Dutch modified cell culture medium buffered with 20 mM HEPES and supplemented with 10% fetal calf serum (FCS), 1 mM piruvate, 2 mM glutamine, 50 μg/ml gentamicin and 2.5 μg/ml fungizone (all obtained from Sigma, St. Louis, MO, USA). To prepare VHSV for *in vivo* challenges or to infect EPC cell monolayers, 2% FCS, 10 mM Tris pH 8.0 and no CO_2 atmosphere were employed. To assay for VHSV infectivity, cleared supernatants from VHSV-infected EPC cell monolayers were centrifuged at 60,000 × *g* for 180 min at 4 °C, and pellets resuspended in 50 μl aliquots in cell culture medium and frozen at −70 °C until used (Chinchilla et al., 2013).

2.2. Zebrafish maintenance, immunization and harvest of plasma

Adult "extra-large" (XL) zebrafish weighting 700–900 mg (~4 cm in length) were obtained from a local pet shop (Aquarium Madrid, Madrid, Spain). They were maintained at 24–26 °C in 30 L aquaria provided with biological filters, and fed with a commercial diet.

To obtain zebrafish surviving three consecutive VHSV infections, a previously described protocol was followed (Chinchilla et al., 2013). During VHSV-induced mortalities, fish were monitored 2–4 times daily and those with external hemorrhages killed by an overdosage of methanesulfonate 3-aminobenzoic acid ethyl ester (MS-222, Sigma). Animals were handled in accordance with the National and European guidelines and regulations on laboratory animals care. Animal work was approved by the Ethic Committee of the Instituto Nacional de Investigaciones Agrarias (authorization CEEA 2011/022).

Two months after the last VHSV challenge, the zebrafish were anesthetized prior to handling by immersion in 90 mg of MS-222 per liter. Anesthetized zebrafish were bled by cutting the final end of their tails. Blood was collected in 200 μl of sterilized anticoagulant media (0.64 g sodium citrate, 0.15 g EDTA, 0.9 g sodium chloride per 100 ml of water). The diluted individual blood samples were immediately centrifuged at 1000 × *g* for 3 min to obtain the supernatant plasma. Protein content of plasma was evaluated by nanodrop absorbance at 280 nm. Plasma was de-complemented to avoid individual complement interferences with the neutralization assay by heating to 45 °C for 30 min and kept frozen at −20 °C until used (Chinchilla et al., 2013).

2.3. Optimization of recovery of EPC cells in suspension from fixed-permeabilized monolayers (FIXPERM) by trypsin digestion

EPC cells (50,000 cells per well) were plated in poly-D-Lys 96-well plates (Corning, New York, NY, USA) to form the cell monolayers with increased adherence to the solid-phase and incubated overnight at 28 °C. Monolayers were then fixed and permeabilized during 20 min by using one of these treatments: (i) Methanol at −20 °C; (ii) 50% Methanol in phosphate buffered saline (PBS); (iii) 0.1% Glutaraldehyde in PBS; (iv) 1% Glutaraldehyde in PBS; (v) 0.5% Cytofix in PBS; (vi) Cytofix, 4% formaldehyde (without dilution); (vii) 4% Cytofix and 0.1% Triton X-100; (viii) 4% Triton and 0.1% Tween 20; (ix) 4% Cytofix and 0.05% of Saponin; (x) 10% Formaldehyde and 0.1% Triton X-100 in PBS; (xi) 10% Formaldehyde and 0.1% Tween 20 in PBS and (xii) 10% Formaldehyde and 0.05% of Saponin in PBS. Methanol, Glutaraldehyde, Formaldehyde, Triton X-100 and Tween 20 were obtained from Sigma (St. Louis, MO, USA). Cytofix was obtained from BD-Biosciences Pharmingen (San Diego, CA, USA). Saponin (Quil-A) was obtained from Superfos (Vedbaek, Denmark). Cells were then detached from the monolayers by 5 min digestion with 0.25% trypsin, 0.05 M EDTA in RPMI medium by pipetting up and down until no cells remained in the solid-phase as shown by observation with an inverted microscope. Cells suspensions were then counted and cell recovery calculated by the formula, 100 × number of cells in suspension/50,000.

2.4. Preparation of possible contaminants of zebrafish plasma

Because zebrafish plasma could be contaminated during tail bleeding by mucus and/or hemoglobin, their possible interference with the neutralization assay was assayed. To prepare mucus, 5 zebrafish were consecutively agitated and allowed to move for 1 min into 1 ml of anticoagulant media. The mucus was collected in the supernatant after centrifugation. To prepare hemoglobin, zebrafish blood was obtained by bleeding 5 fish from the tail into anticoagulant media. The blood was centrifuged at 10,000 × *g* 5 min and the pellet containing the red blood cells was lysed in distilled water with the aid of 2–3 sonication bursts. The hemoglobin containing supernatant was then obtained by centrifugation. The protein content of each of the above mentioned extracts was estimated by nanodrop measurements at 280 nm and adjusted to 2 mg of protein per ml. The extracts were kept at −20 °C until used.

2.5. Estimation of VHSV neutralizing antibodies by using FIXPERM

The procedure was modified from our previous microneutralization assay that used focus forming units (ffu) detected after immunostaining of VHSV-infected cells with diaminobenzidine (DAB) (Sigma) (Chinchilla et al., 2013). EPC cells (50,000 cells per well) were plated and grown overnight at 28 °C in 96-well plates of polystyrene coated with poly-D-Lys (Corning, New York, NY, USA) in 100 μl of cell culture medium to form the monolayers. De-complemented zebrafish plasma 20-fold diluted (~40 μg of plasma protein per well) were agitated during 2 h and incubated overnight (~16 h) with 300 ffu of purified VHSV per well in cell culture medium containing 2% FCS, 10 mM Tris pH 8.0 and without CO_2 atmosphere at 14 °C. Cell monolayers were then infected by adding 100 μl of the VHSV-zebrafish plasma mixtures and agitating gently during 2 h at 14 °C. After washing the excess of VHSV by flicking the plate and refilling the wells with cell culture medium containing 2% FCS, 10 mM Tris pH 8.0 and without CO_2 atmosphere, VHSV-infected monolayers were incubated overnight at 14 °C before being fixed with 10% formaldehyde in phosphate buffered saline (PBS) during 20 min.

The fixed monolayers were reversibly permeabilized with 0.05% Saponin, 0.01% N_3Na in PBS during 15 min. To detect the most abundant intracellular nucleoprotein N of VHSV, the monoclonal antibody (MAb) 2C9 (Sanz and Coll, 1992) 500-fold diluted in 2% FCS, 0.05% Saponin, 0.01% N_3Na in PBS (permeabilizing buffer) was added to the wells (100 μl/well) and incubated for 1 h. After washing the cell monolayers with the permeabilizing buffer and manually flicking the plate, 100 μl of rabbit FITC-labeled anti-mouse IgG (Nordic, Tilburg, The Netherlands) was added to each well, and the incubation continued for 30 min. After washing 3 times with the permeabilizing buffer, the monolayers were incubated 5 min with 100 μl of 0.25% trypsin 0.02% EDTA (Sigma, St. Louis, MS, USA). Trypsin digestion was stopped by the addition of 50 μl per well of 1% BSA, 50 mM EDTA, 0.01% N_3Na in PBS. To obtain EPC cell suspensions, the cellular contents of the wells were pipetted up and down several times until most cells were suspended as indicated by observation with an inverted microscope.

The suspended cells were analyzed in a BD FACS Canto II apparatus (Beckton Dickinson, San Agustin de Guadalix, Madrid, Spain) provided with a hightroughput sampler (HTS). Forward (FSC) and side (SSC) scatter threshold values corresponding to damaged cells, cellular debris and/or cellular aggregates (<10% of total events) were eliminated from the analysis by defining an individual cell region. Individual cells were gated for the defined FSC/SSC region and fluorescence. The number of fluorescent cells over a threshold containing 95% (mean + 2 standard deviations) of non-infected EPC cells was then determined by gating a non-infected EPC cell control. An VHSV-infected cell control in the absence of added zebrafish plasma was used to confirm that 40–60% of the cells were infected. After the multiwell autosampler gated 10,000 events per well of the 96-well plates (30–60 min), the automatic batch analysis feature of BD FACSDIVA calculated the percentage of fluorescent (N-positive, VHSV-infected) cells by the formula, 100 × number of cells with fluorescences above the threshold/total number of cells gated per well. The software exported all results to an MS Excel sheet for additional calculations. Linear fitting strength was estimated by calculating the Pearson's correlation coefficient by using the Origin program (OriginPro 8.5.1. SR, Northampton, MA, USA). Comparison of values of percentage of fluorescent cells were performed by using the Student's *t*-test at the $p < 0.05$ level.

3. Results

3.1. Optimization of fixation-permeabilization of monolayers of EPC cells

To differentiate non-infected from VHSV-infected cells by using flow cytometry analysis, the cells need to be in suspension rather than in monolayer. However, preliminary analysis showed that intracellular immunostaining of cells in suspension required numerous washing/centrifugation steps that diminished their recovery, were time-consuming and increased handling errors when processing a large number of samples. To substitute centrifugation steps by "in situ" washing of monolayers, cell adhesion to the polystyrene cell culture surfaces need to be increased. To increase their adhesion, cells were plated, grown, and fixed to poly-D-Lys coated plates. Because antibodies need to get through the fixed cellular membranes to stain intracellular VHSV N nucleoprotein, continuous permeabilization was required during the immunostaining procedure. However, to suspend the cells by trypsin before flow cytometry, cells need to be non-permeable to minimize digestion of intracellular antibody-N complexes. Therefore, different methods to balance conditions of fixation/permeabilization/trypsin-digestion of poly-D-Lys-plated EPC cell monolayers were tested first to search for the best recovery of cells in suspension.

Fig. 1 shows that the highest recovery of EPC cells in suspension obtained after trypsin digestion with different fixing and permeabilizing agents was ~80%. It was obtained with 10% formaldehyde and 0.05% Saponin. Lower but still significant cell recoveries could be obtained by using Cytofix or Formaldehyde fixation with either Tween 20 or Saponin as permeabilizing agents. In contrast, by using Methanol, Glutaraldehyde and Cytofix or Formaldehyde with Triton X-100, cell recoveries were much lower (10–20%). When using Methanol as fixative/permeabilizer, no cells remained in the solid-phase due to their lysis, as confirmed with the inverted microscope. In contrast, when using Glutaraldehyde, no cells could be detached with trypsin since most of the cell monolayer remained intact as confirmed with the inverted microscope. Therefore, Cytofix and Formaldehyde were selected as potential fixing agents while Saponin was selected as the permeabilizing agent for further experiments.

3.2. Immunostaining of the VHSV N nucleoprotein in fixed-permeabilized monolayers (FIXPERM) of EPC cells

In preliminary experiments, 100 ffu of VHSV per 50,000 EPC cells were used for infection since that multiplicity yielded optimal numbers of ffu per well for counting with the microscope (Chinchilla et al., 2013). The high titer anti-N MAb 2C9 was selected for immunostaining since it showed lower backgrounds than other MAbs or polyclonal antibodies raised in rabbits (data not shown). However, under those infections conditions, when cells were fixed-permeabilized with Cytofix and immunostained in the absence of permeabilization, high backgrounds were obtained after flow cytometry, as shown by the small differences between non-infected (white histogram) and VHSV-infected (gray histogram) cells (Fig. 2A). Those preliminary results suggested that some of the antibodies used for the immunostaining procedure remained intracellular despite the washing steps. In contrast, experiments

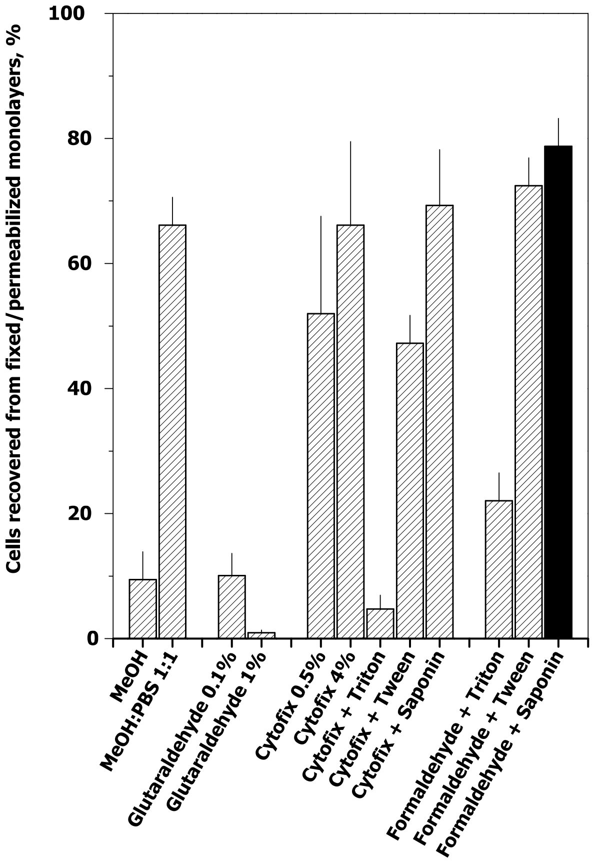

Fig. 1. Recovery of cells in suspension from fixed-permeabilized monolayers by trypsin digestion. EPC cells were plated in poly-D-Lys 96-well solid-phase wells (50,000 cells per well) at 28 °C. After one day, cell monolayers were fixed and permeabilized by different treatments. Cells were then detached by digestion with 0.25% trypsin, 0.02% EDTA and pipetting up and down until no cells remained in the solid-phase. Cells in suspension were then counted and recovery calculated by the formula, 100 × number of cells/50,000. (i) Methanol, −20 °C. (ii) Methanol:PBS 1:1, 50% methanol in phosphate buffered saline (PBS). (iii) Glutaraldehyde, 0.1% in PBS. (iv) Glutaraldehyde, 1% in PBS. (v) Cytofix, 0.5% in PBS. (vi) Cytofix, 4% (without dilution). (vii) Cytofix + Triton, 4% Cytofix and 0.1% Triton X-100. (viii) Cytofix + Tween, 4% Cytofix and 0.1% Tween 20. (ix) Cytofix + Saponin, 4% Cytofix and 0.05% of Saponin. (x) Formaldehyde + Triton, 10% Formaldehyde and 0.1% Triton X-100 in PBS. (xi) Formaldehyde + Tween, 10% Formaldehyde and 0.1% Tween 20 in PBS. (xii) Formaldehyde + Saponin, 10% formaldehyde and 0.05% of Saponin in PBS. Black bar, treatment showing the highest cell recovery.

using cell monolayers fixed with 10% Formaldehyde and then permeabilized with 0.05% of Saponin, and immunostained in the continuous presence of Saponin, increased the differences of mean fluorescences between non-infected and VHSV-infected cells to ∼1–2 logs (not shown). When the multiplicity of infection was increased 3-fold, a separation of ∼2–3 logs between peak fluorescences of non-infected and VHSV-infected cells were obtained ($n = 17$ experiments) (Fig. 2B). The recoveries of cells in suspension after VHSV infection, immunostaining, trypsin digestion and flow cytometry with respect to the original number of plated cells were of 35.4 ± 6.7% ($n = 95$). Most of the recovered cells remained intact as shown by the corresponding FSC/SSC dot plots (Fig. 2C). Fig. 3 schematizes the final protocol for the FIXPERM micro-neutralization assay.

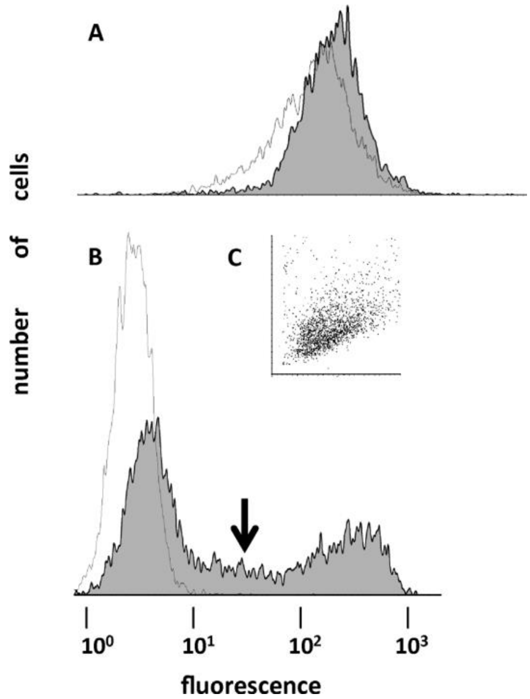

Fig. 2. Flow cytometry of non-infected and VHSV-infected EPC cells fixed-permeabilized and immunostained with anti-VHSV N monoclonal antibody. VHSV was added to EPC cell monolayers and incubated overnight. VHSV infection was stopped by fixation/permeabilization with Cytofix (A) or with 10% formaldehyde and then 0.05% Saponin (B). EPC cells were immunostained with anti-VHSV N nucleoprotein and FITC labeled anti-mouse IgG by using the cells in the absence of permeabilization (A) or in the continuous presence of Saponin (B). Fluorescence profiles of non-infected (white histogram) and VHSV-infected (gray histogram) EPC cells. (C insert) dot plot of forward/side (FSC/SSC) scattering of the EPC cells from assay B. Vertical arrow, threshold between non-infected and VHSV-infected EPC cells calculated by the mean of fluorescences in non-infected cells (white histogram) plus 2 standard deviations to contain 95% of the cells.

3.3. Correlation between focus forming unit (ffu) and fixed-permeabilized monolayer (FIXPERM) assays

A dose-response curve showing linear correlation with a Pearson's coefficient of $r = 0.99$ was obtained between the percentage of fluorescent cells determined by FIXPERM assays and DAB-stained ffu determined by ffu assays at different concentrations of VHSV (Fig. 4A). Thus, by infecting 50,000 cells with 300 ffu of VHSV per well, 40–60% of the cells were fluorescent (fluorescence values above the threshold) in different experiments ($n = 8$). By increasing VHSV to >700 ffu per well, >90% of cells were fluorescent and ffu was outside the range of counting (Fig. 4A). According to these data, 200–500 ffu of VHSV per 50,000 cells was the optimal range required to carried out the FIXPERM micro-neutralization assays.

3.4. Determination of neutralizing antibodies in plasma from zebrafish surviving VHSV infection

To assess for blood neutralizing antibodies, zebrafish surviving three consecutive VHSV infections were individually bleed by cutting their tails into anticoagulant medium. By using this method, a concentration of 2.6 ± 1.2 mg of protein per ml ($n = 64$) of diluted plasma was obtained. Because zebrafish plasma obtained by bleeding from their tails could be contaminated by mucus from the skin and/or hemoglobin from exceptional red blood cell haemolysis, zebrafish mucus and hemoglobin were obtained and assayed for their possible interference with the FIXPERM micro-neutralization

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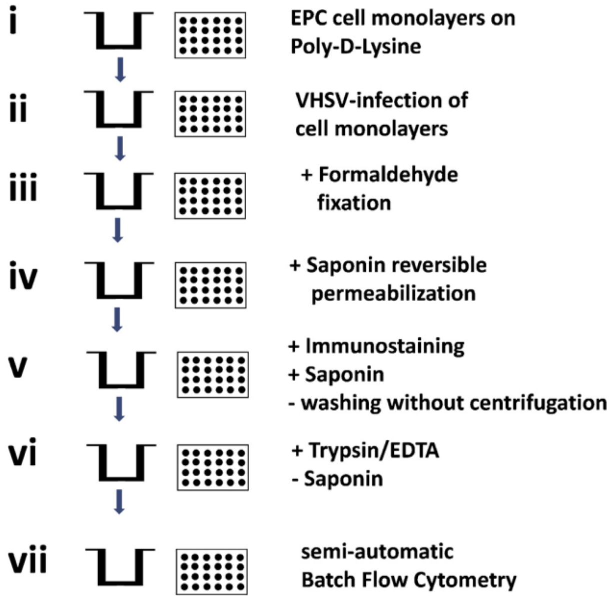

Fig. 3. Scheme of the final design of the FIXPERM method. The steps were: (i) EPC cell monolayers were obtained by plating 50,000 EPC cells per well in 96-well plates coated with poly-D-Lys. (ii) The monolayers were incubated overnight with 300 ffu per well of purified VHSV after previous overnight incubation of VHSV with plasma from VHSV immunized zebrafish. (iii) The monolayers were fixed to the poly-D-Lys solid-phase with formaldehyde. (iv) The monolayers were permeabilized with Saponin. (v) The intracellular immunostaining of the N nucleoprotein of VHSV was performed in the presence of Saponin and washing the monolayers by manually flicking the plates. (vi) The cells were suspended from the fixed-stained cell monolayers by digestion with trypsin-EDTA in the absence of Saponin. (vii) The suspended cells were gated with the hightroughput sampler (HTS) of the BD FACS Canto II and analyzed by the Batch Analysis feature (BD FACSDIVA software).

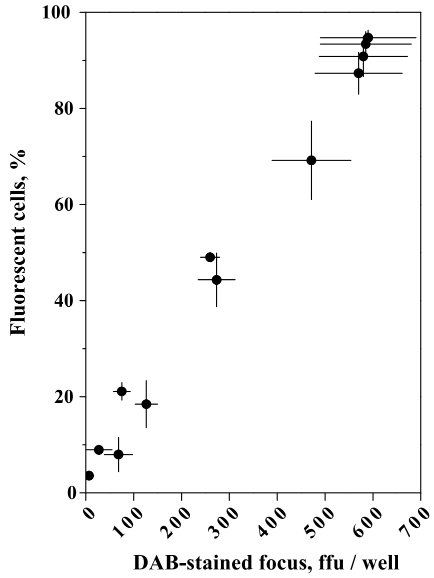

Fig. 4. Relationship between micro-neutralization assays counting ffu (DAB-stained cells) and and FIXPERM (fluorescent cells) at different VHSV dosages. Plasma samples were incubated with VHSV and the mixtures assayed by the focus forming unit (ffu) (Chinchilla et al., 2013; Lorenzo et al., 1996) and by the FIXPERM micro-neutralization assays. The ffu were estimated by DAB-staining and their numbers per well counted with the aid of an inverted microscope ($n = 4$). The percentage of fluorescent (VHSV-infected) EPC cells after flow cytometry were calculated by the formula, number of fluorescent EPC cells in the presence of plasma/number of cells gated. Means and standard deviations ($n = 4$) were represented.

assay. However, nor mucus nor hemoglobin showed any interference with the assay at 40 μg of protein per well, the maximal plasma protein concentration used for the assay (data not shown).

Results obtained from different FIXPERM micro-neutralization assays of plasma samples selected in a wide range of percentage of fluorescent cells, showed a linear correlation of duplicate determinations with a Pearson's correlation coefficient of $r = 0.92$ (Fig. 5A). Fig. 5B shows the distribution profiles of the number of individual zebrafish plasma in function of their percentage of fluorescent cells

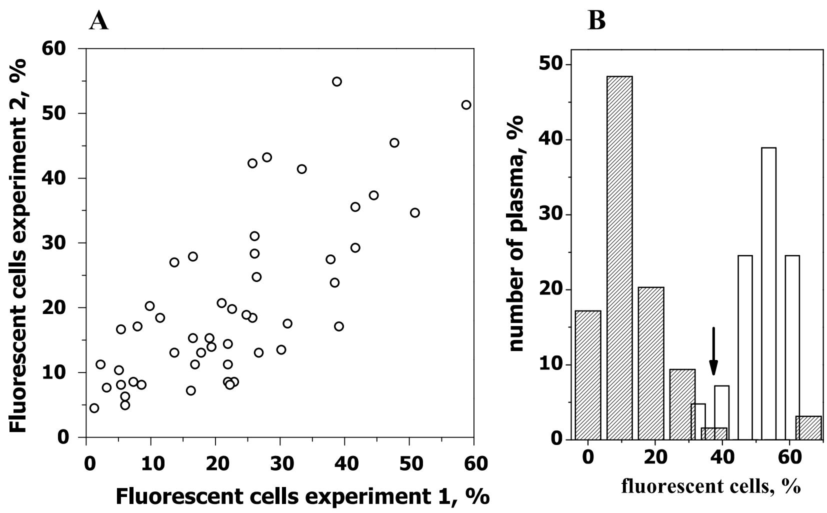

Fig. 5. Distribution of the numbers of zebrafish plasma from non-infected or survivors of VHSV infections assayed by the FIXPERM micro-neutralization assay. (A) Comparison of percentage of fluorescent cells from 57 selected plasma samples from non- infected and survivor zebrafish assayed in two different experiments. Each point corresponds to an individual zebrafish. (B) Comparison of distribution of plasma frequencies of percentage of fluorescent cells from non-infected zebrafish (white bars, $n = 167$) and from zebrafish surviving VHSV infections (hatched bars, $n = 64$). Vertical arrow, threshold of values significantly lower than the mean percentage of fluorescent cells from plasma from non-infected zebrafish at the $p < 0.05$ level (Student's t-test).

from non-infected (hatched bars) and from survivors (white bars) of VHSV infections. The average percentage of infected cells obtained from plasma from non-infected zebrafish were 51.8 ± 7.3% (n = 167) while those from VHSV survivors were 13.0 ± 10.6% (n = 64). Up to 95.1% of the VHSV survivors had significatively higher neutralizing antibodies in their plasma than non-infected zebrafish at the $p < 0.05$ level (Student's t-test) (Fig. 5B).

4. Discussion

A micro-neutralization assay to estimate antibodies to viruses has been developed by using fixed-permeabilized monolayer (FIXPERM) cells for high-throughput flow cytometry analysis. The FIXPERM micro-neutralization assay was used to estimate viral neutralizing antibodies levels in plasma from zebrafish (*Danio rerio*) surviving VHSV infections. Neutralization activity could be detected in the low amounts of blood that could be obtained from individual zebrafish by tail bleeding (corresponding to ~3 μl of blood per zebrafish). The procedure was applied to a large number of individual samples, being capable of reading the results in 30–60 min per 96-well plate, only one day after infection of the cell monolayers.

Due to the absence of any well characterized zebrafish IgM reagents, the detection of VHSV-neutralizing activity is a first approach to study and demonstrate their antibody responses to viruses (Chinchilla et al., 2013). Therefore, the development of a high throughput assay to detect neutralizing antibody levels in zebrafish would increase the use of this fish as an infectious disease model. By using this method, neutralization of VHSV could be demonstrated in 95.1% of plasma from survivors of VHSV infections. In contrast, only 40–50% of the sera from rainbow trout (*O. mykiss*) surviving a VHSV infection showed anti-VHSV neutralizing antibodies by pfu assays (Olesen and Jorgensen, 1986).

For fixing cell monolayers other authors used formaldehyde at concentrations of 0.5% (Grabner et al., 2000) or 3% (Gerner et al., 2008). In the present method, a higher formaldehyde concentration (10%) and poly-D-Lys coated plates were used to increase cell adherence. Increased adherence minimized cell losses during subsequent immunostaining/washing steps allowing the use of a small number of cells in 96-well plates for flow cytometry. To obtain around 1 log difference between non-infected and infected cells, published flow cytometry methods to detect intracellular viruses employed Triton X-100 to permeabilize fixed cells (Kao et al., 2001). However, 2–3 log differences could be obtained in this work by using Saponin to permeabilize fixed cells, confirming that Saponin was the best permeabilizing agent (Gerner et al., 2008). Further to the reports mentioned above, other described methods for instance those using green fluorescent protein recombinant viruses to facilitate neutralization assays, required centrifugation steps for immunostaining before flow cytometry too and could benefit from the FIXPERM method (Earl et al., 2003).

Therefore, among the advantages of the FIXPERM micro-neutralization assay are the possibilities to: (i) carry out all immunostaining steps for multiple samples in 96-well plates minimizing the use of blood plasma and reagents, (ii) wash reagents during immunostaining without centrifugation steps, (iii) perform viral infections overnight, (iv) analyze fluorescent cells semi automatically, and (v) output data automatically to an Excel spread sheet ready for additional calculations. In conclusion, fixed-permeabilized monolayers (Gerner et al., 2008; Grabner et al., 2000) of cells were combined with micro-incubation of viral and host antibodies (Chinchilla et al., 2013; Lorenzo et al., 1996) and centrifugation-free immunostaining, to obtain semi automatic processing of large number of samples to estimate neutralizing antibodies by multiwell flow cytometry.

The FIXPERM micro-neutralization assay may be useful to assay for antibodies to other viruses. However, because no particular fixation/permeabilization protocol is ideal for all types of cell monolayers and/or viruses, some optimization for detection of the main protein antigen for each particular case might be required.

Acknowledgements

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IV.GLOBAL DISCUSSION

Preface to discussion

Non-virion (NV) proteins from different novirhabdoviruses are dispensable but necessary for an efficient virus replication and essential for virus pathogenicity, except for SHRV. These proteins are important to favour virus replication due to inhibition of immune response mechanisms and cell survival. Despite dissimilar primary NV protein sequences among VHSV, IHNV and HIRRV, these proteins produce analogous inhibitory effects. It is unknown whether this might be related to linear polypeptide sequences or due to a more complex tertiary protein structure, which is not shared by SHRV NV. In this part of the thesis, we discuss the contribution of different NV regions and individual amino acids from the region between the two first α-helices (hinge region) to VHSV NV function and its subcellular location [261]. Here, it is also discussed the data obtained and presented in this thesis from rNV-driven transcriptional changes in rainbow trout, by using immune-targeted microarrays [257].

A. CELLULAR LOCATION OF NV PROTEINS AND ITS RELATION WITH FUNCTION

The intracellular localization of NV proteins was first observed by immunofluorescence studies performed by Schütze et al. 1996 [97]. To this end, RTG and EPC cells were infected with VHSV and IHNV, respectively, and reaction with the corresponding anti-NV sera resulted in a diffusely distributed and perinuclear cytoplasmic fluorescence into the cells for VHSV but a nuclear staining for IHNV [97]. However, these observations were not pointed out by the authors. In addition, they observed a similar recognition pattern in infected cells when using polyclonal antibodies against virus, which was defined as a strong reaction pattern with coarse cytoplasmic granuloid structures in VHSV- or IHNV-infected cells [97].

Several years after those findings, through plasmid transfection of IHNV NV (PRT strain) fused to GFP, Choi et al. 2011 [238] also demonstrated that the NV protein of IHNV localizes to the nucleus and identified a nuclear location signal (NLS) sequence within the protein, consisting in four amino acid residues (^{32}EGDL^{35}) and located between the two first α-helices (termed by us “hinge region”), as responsible for its nuclear localization. This NLS was also demonstrated to be essential for a suitable function of the protein and viral replication [238]. Recently, other authors generated a recombinant IHNV virus with a NV-ΔEGDL gene. This rIHNV-NV-ΔEGDL gave rise to lower pathogenicity, and hence viral titers, and also more interferon and IL-6 levels in host compared to wild-type IHNV [248]. Therefore, it is well established the nuclear location of IHNV NV. Similarly, we have confirmed the nuclear location of IHNV NV from different strains, PRT strain (data not shown) and Oregon69 strain **(Figure 6, Paper II)**, and determined that the NV of HIRRV is also located at nucleus and contains a similar NLS motif at the hinge region. Even though the amino acid sequence of the NLS is highly conserved among IHNV strains (especially amino acid residues ^{33}GDL^{35}), the responsible NLS sequence for HIRRV NV nuclear location differed from the former ones (^{31}DDGKI^{35} for HIRRV NV) [261].

In the present work, we have also described a cytoplasmic location pattern for VHSV NV 07.71 **(Figure 6, Paper II)**, with certain perinuclear accumulation in a similar way to that observed by Schütze et al. 1996 for VHSV Fil3 strain [97]. The comparative sequence analysis between the hinge regions of VHSV and IHNV NV proteins showed very few identities, suggesting the lack of a canonical or non-canonical NLS compared to IHNV NV and HIRRV NV. As an approach, we carried out the insertion of the NLS (EGDL motif) of IHNV NV from PRT strain into the hinge region of the VHSV NV 07.71, between residues LNCDF and DRSD after amino acid 37, but this modification was not enough to redirect VHSV NV-EGDL to the nucleus, thus, remaining at cytoplasm unlike IHNV NV PRT.

Very recently, it has been described that both NV from VHSV and IHNV interact with protein phosphatase, 1Bb (PPM1Bb), which is a member of the PPC2 family and which dephosphorylates TBK1 [258]. Nuclear (IHNV) and cytosolic (VHSV) NV proteins seem to interact with PPM1Bb in a different manner; VHSV NV recruits PPM1Bb around mitochondria to dephosphorylate TBK1, and further interferes with the retinoic acid inducible gene-I (RIG-I) response, whereas IHNV NV could bind to PPM1Bb around mitochondria and nucleus, acting on nuclear substrates different from TBK1 for inhibiting RIG-I response [258].

Finally, in the case of snakehead rhabdovirus (SHRV) NV protein, we also observed a cytoplasmic location for the NV (unpublished data). Nevertheless, this fact could not be correlated with any function **(Figure 4A, Paper II)**, which is still unknown for NV of SHRV [262], nor the higher degree of identity among VHSV and SHRV NV proteins (23 % of identity, see C.1 section) compared to other novirhabdoviruses. On the other hand, dissimilarity is not a good predictor for function because IHNV NV and VHSV NV only share 15%-16% of identity and they both have conserved the inhibitory function described for NV proteins. Regarding whole genome homology, identity values are comparable among novirhabdoviruses, since the genome comparison of Great Lakes VHSV strain (MI03GL) with SHRV exhibits a 62% of identity, whereas whole genomes from IHNV and HIRRV compared to that of VHSV showed a 56% of identity [99].

B. VHSV NV REGIONS INVOLVED IN FUNCTION AND LOCATION

As previously discussed, the peptide identified as a nuclear location signal (NLS) sequence within the region located between the two first α-helices (termed by us the "hinge region") in the IHNV NV (PRT) demonstrated to be very important for IHNV NV location and function [238, 248]. Given that this NLS is mapped at this site within IHNV NV molecule and the homologous VHSV NV region could be important for function, we carried out functional studies using VHSV NV mutants and fragments to determine their location and activity **(Figures 1 and 2, Paper II)**. No previous similar studies were carried out with VHSV NV protein. The results obtained suggested that tertiary structure is important for NV function since F1 retained its ability to inhibit *mx* and *Il8 in* zebrafish (ZF4) cell line, being F1 the fragment with more α-helixes **(Figure 3, Paper II)**. On the contrary, F2 fragment lost the capacity to inhibit *mx* and *il8*, being F2 the C-terminal fragment with β-sheets **(Figure 3, Paper II)**. The N-terminal part of NV (i.e.: fragment F1) induced a similar *mx* and *il8* modulation to wt-NV and also shared the same cytoplasmic location. Fragments F3 to F8, containing at least one of the two first α-helices and/or the hinge region, could be enough for maintaining an analogous activity to the complete NV but not the subcellular distribution **(Figures 1 and 3, Paper II)**. Thus, fragment F6 induced similar *mx* and *il8* levels to those of F1 and wt-NV but did not share the same location pattern **(Figures 3 and 8, Paper II)**, being more diffusely distributed in the nucleus and cytoplasm. This data suggests that the third α-helix absent in F6 could be important for a better tertiary structure and a suitable location. The NV function has been related to its location and interaction with mitochondria [258]. More studies are needed to confirm that F1 but not F6 could interact with mitochondria. On the other hand, the C-terminal part of NV (i.e.: fragment F2) plays an unknown role in the cell; it upregulated *mx* and *il8* transcript levels when compared to whole NV and did not have the characteristic cytoplasmic location pattern of the NV protein and the F1 fragment [261]. Altogether, our data suggested that F1, and not shorter N-terminal fragments nor F2, maintained a proper cytosolic location for a possible and suitable interaction with PPM1Bb in the close vicinity of mitochondria. This would need to be confirmed in future experiments.

In order to study the contribution of amino acids from the hinge region to *mx*- and *il8*-induced transcript levels and location, we studied eleven VHSV NV-derived mutants at positions 28, 31 and 33-41 **(Figure 2, Paper II)**, changing each corresponding amino acid to alanine. These amino acid positions were mainly chosen based on the existence of conserved amino acid residues into the VHSV NV protein of different strains (07.71 and Fil3) and shared amino acids between NVs from different novirhabdoviruses (VHSV and IHNV).

With regard to cellular location, VHSV NV 07.71 and all NV mutants studied exhibited a cytoplasmic distribution and shared this characteristic with VHSV NV Fil3 strain, as discussed above. Surprisingly, while mutants NV28 (D28A), NV31 (N31A), NV33 (L33A), and NV37 (F37A) induced similar *mx* and *il8* levels to wt-NV, the mutants NV36 (D36A), NV39 (R39A), and NV41 (D41A) induced a stronger *mx* and *il8* downregulation than wt-NV **(Figure 4, Paper II)**. The later data could suggest a better stabilization of the NV molecule in those three mutants and an improved interaction with its molecular target PPM1Bb that would induce a stronger TBK1 dephosphorylation, which in turn would lead to a lower expression of *mx* and *il8* transcript levels. Among the available VHSV NV amino acid sequences, there is no alanine at neither of the positions changed; residues at positions 36 and 39 are conserved whereas amino acids are variable at position 41 (aspartic acid or glutamic acid). It is interesting to note that a mutation incidentally conducting alanine at positions 36, 39 or 41 in the NV (07.71) protein might produce a more pathogenic VHSV strain **(Table 1, Paper II)**. On the other hand, we obtained three-dimensional models of the wild-type VHSV NV 07.71 protein, and mutants NVD36A, NVR39A, and NVD41A (by using 3Dpro software, Baldi group server, http://scratch.proteomics.ics.uci.edu). These predicted structures did not show any common molecular characteristic for correlating with their improved function **(Figure 5, Paper II)**.

On the other hand, certain NV-derived mutants (N34A, C35A, D38A and S40A) lost their ability to downregulate *mx*, but not *il8*, compared to the wt-NV protein **(Figure 4, Paper II)**. Altogether, these data suggest that a single mutation in the NV could lead to a loss or an increase of function that would impact the virulence phenotype of the virus. In that sense, recently Baillon et al. 2017 [263] observed that various attenuated isolates of VHSV naturally bear a serine at position 116, except HRV2 isolate that despite bearing serine maintains its virulence. They replaced the arginine by serine at position 116 (R116S) in NV 07.71 and generated a virus by reverse genetics. This rVHSV NV (R116S) derived from a virulent VHSV strain was highly attenuated and those surviving fish were almost completely protected (80% survival) from a challenge with the wild-type VHSV. Therefore, these results showed that the presence of serine at position 116 affects to NV stability or structure for the correct function of NV 07.71. However, HRV2 isolate is virulent and naturally bears S116, suggesting that other amino acids along the protein are important for maintaining correct function.

Finally, further research on different VHSV NV proteins using reverse genetics is needed to confirm whether the same mutations (e.g. D36A, R39A, and D41A) and/or other positions would lead to an improved inhibition similar to that observed in this study with VHSV NV 07.71 or the opposite. In addition, future studies would help to clarify the mechanisms implied in the interaction of the wt-NV and mutants with PPM1Bb.

Despite the different primary protein sequences among IHNV and VHSV NV proteins, here we demonstrated that the VHSV NV homologous region (hinge region) is also important for function, despite the absence of NLS.

C. INTERFERENCE OF VHSV NV WITH THE HOST IMMUNE RESPONSE

The NV protein of VHSV has been implicated in the interference of some host innate mechanisms *in vitro*, but little is known about the transcriptional changes induced by the protein *in vivo*. For this purpose, trouts were intraperitoneally injected with soluble recombinant NV (rNV) and the transcriptome changes induced by the protein were analysed at 48 h post-injection **(Figures 1 and 2, Paper I)**. Overall, our main results showed that rNV induced the inhibition of multiple innate and adaptive immune-related genes **(Figure 3, Paper I)**. Although adaptive immune response begins later than the innate and the duration of the experiment, these results could be explained by the observed downregulation of some innate genes involved in triggering adaptive immune responses such as interleukins, interferons and transcription factors. The loss of the innate immune response and the delay of the adaptive immune response induced by the rNV protein would favour viral replication at least during the early stages of VHSV infection in a context of weak antiviral state. To our knowledge, this is the first report suggesting most important possible rNV ligands and describing extensive transcriptional *in vivo* profile changes induced by the rNV protein from any novirhabdovirus [257]; and probably has been taken as a reference for further studies on NV targets [258].

1. Confirming previous results of VHSV NV

Gene expression changes in rainbow trout modulated by soluble rNV confirmed previous results on interferon (*ifn*) and ifn-related genes (*mx*) [199, 264], caspase-mediated apoptosis [237] and TNF-mediated NF-kB activation, increasing the confidence in the rest of the novel data obtained from the immune-targeted microarray.

a. NV inhibits the IFN system

Interferons (IFNs) are the hallmark of vertebrate innate immunity to viruses. They belong to the cytokine family and can be classified into three families in mammals (type I IFNs, which include IFNα and IFN-β, type II or IFN-γ, and type III or IFN-λ) based on their structural and functional properties, and on their receptors [265]. Both type I and type III IFNs are strongly induced by viral infections and play a major role in the early innate response against viruses [265]. In contrast, type II IFNs (IFN-γ) are immunoregulatory cytokines also present in fish [266], with key roles in both innate and adaptive immunity [265].

Although fish IFNs have been often compared to type I mammalian IFNs, their expression is subject to an additional level of regulation [267]. Fish IFN promoter is typically made of two regions, and an alternative use of initiation of transcription leads to different transcripts. Upon viral infection, an alternative IFN isoform with an effective signal peptide is activated (secreted isoform), while the normal promoter usage leads to the expression of a non-secreted isoform in absence of stimulation [268] [185] [203]. Nevertheless, while the function of the long transcripts leading to non-secreted IFN was initially elusive [185], it was later observed that their overexpression leads to induction of orthomyxovirus resistance gene (*mx)* and to a protection against viral infection [269]. As overexpression of both intracellular IFNs and receptors leads to STAT signaling (JAK/STAT pathway) and induction of resistance to viral infection, it is proposed that they act as a partly constitutive cellular system of antiviral defence [269].

Mammalian type I IFNs bind the IFN-alpha/beta receptor subunit 1/2 (IFNAR1/IFNAR2) complex, and signal through the JAK/STAT pathway involving Jak kinases Jak1 and Tyk2 and STAT transcription factors (STAT-1 and -2, principally). Cellular response to IFN then consists of the up-regulation of a large number of genes, named IFN stimulated genes (ISG). While some ISG are modulators of the signaling pathways upstream or downstream of IFN itself (e.g. via IRF1), others ISG have a direct antiviral activity, such as Mx, illustrating the diversity of the cellular pathways triggered by viral infections and the functional complexity of type I IFNs [265, 267]. This IFN I promotion of an antiviral state through ISG such as Mx has been used to monitor IFN activity in trout [253] and has also been considered as a marker of the inhibitory activity of NV in this thesis [261].

On the other hand, MX proteins are large GTPases involved in intracellular membrane remodelling and intracellular trafficking [270] that have been cloned and characterised in many fish species [264, 267]. However, evidence for antiviral activity has only been established for Mx proteins from Japanese flounder and Atlantic salmon [271, 272]. In rainbow trout, the target of our study, three different Mx genes (*mx*1, *mx*2, and *mx*3) have been previously cloned and characterized by Trobridge et al. 1995, 1997 [273, 274]. Over-expression of Mx in rainbow trout cells did not inhibit IHNV infection [273]. However, Mx was up-regulated during the early interferon response after DNA vaccination against VHSV [85], which was correlated to early protection [214]. Interestingly, different expression patterns were found for the three Mx isoforms after VHSV infection or stimulation with poly I:C, depending on the tissues, which suggested different regulation pathways [264].

The importance of IFN system has also been reflected in our study, given that based on the modulation of these genes (*mx, ifn* or *isg* genes) we chose the most suitable control (PolyH) to study the effects of rNV. In this work **(Paper I, [257])**, we found multiple probes of type-I interferon genes (*ifn1-5*) and *ifng* downregulated by rNV as well as all the myxovirus resistance GTPase genes (*mx1–mx3*) **(Figure 4, Paper I).** Our results confirmed previous comparative studies between wild-type and NV knockout VHSV in which demonstrated that NV protein may downregulate host *ifn1*/*mx* transcriptional levels during *in vitro* infection of cyprinid-derived cell lines (EPC) and *in vivo* infection of olive flounder, suggesting that NV interferes with IFN defences to favour VHSV replication [251]. In addition, Choi et al. 2011 [238] observed similar effects in the virus-induced IFN response *in vitro* but with the NV of IHNV, and Wu et al. 2018 observed similar data with a recombinant IHNV expressing a NV without NLS motif (EGDL peptide) *in vivo* [248]. Although IHNV and VHSV NV proteins do not share any significant identity, Thoulouze et al. 2004 [240] showed that both NV proteins play a similar role since the replacement of IHNV NV by that of VHSV, rIHNV-NV_{VHSV}, produced the same replication levels *in vitro* as in the wild-type IHNV, probably via inhibition of host IFN system as IHNV NV does [238]. Because signal transducer and activator of transcription (STAT1) is a cytoplasmic latent transcription factor that becomes activated by some IFNs (i.e. IFNγ and IFN1) and binds to specific transcription factor binding sites (TFBS) to increase IFNs and IFN-related genes expression (such as *mx*), the downregulation observed suggests that NV not only interferes *in vivo* with the initial inflammatory and antiviral response to favour VHSV replication but also with the adaptive arm of immunity (i.e. IFNγ).

b. NV inhibits apoptosis

Apoptosis, or programmed cell death, is an important physiological process for host defense against viral infection carried out by caspases, and it usually occurs at the early stage of viral infection, thus limiting viral propagation [275, 276].

Many members of the *Rhabdoviridae* family are known to induce apoptosis. In case of novirhabdoviruses, it was shown that *in vivo* and *in vitro* infection with VHSV [237, 277-279] and *in vitro* infection with IHNV [250] results in extensive induction of apoptosis and that matrix (M) protein of novirhabdovirus would be involved in that induction [138-140]

As with other viruses, VHSV suppresses or evades components of the host antiviral responses in order to propagate. To overcome host resistance, many viruses carry antiapoptotic factors to inhibit apoptosis (e.g. influenza virus, hepatitis C virus, etc.). Studies based on NV-deficient and NV-knockout recombinant viruses have suggested that NV protein (and not the gene or the mRNA) suppresses apoptosis at early stage of infection (before 24 hpi) in cell culture [237, 250]. These recombinant viruses caused greater apoptotic effects than wildtype (rVHSV), as shown by stronger DNA laddering [237, 279], higher activity of caspases (initiator caspases 8 and 9, and effector caspase 3 were significantly induced at 16-20-24hpi) [237] and sensitivity to apoptotic inducers (Actinomycin D) [237, 279]. Similarly to Ammayappan 2011, we observed downregulation of caspases 9 and 3 by rNV injection in trout, and also described new caspase transcripts downregulated such as *casp1a* and *casp6* [257] **(Figure 4, Paper I)**. Moreover, this antiapoptotic function of NV has been demonstrated to be restored by the NV of other related novirhabdoviruses despite the low homology exhibited between them [237].

It is very interesting that novirhabdoviruses produce of a large amount of matrix (M) protein, which induces apoptosis, and at the same time express catalytic amounts of NV protein to suppress apoptosis. According to Ammayappan and Vakharia (2011) [237], one possible explanation is that NV protein is produced in very small amounts to prevent early apoptosis (lengthen the time for viral replication in the cells) and is not produced enough to thwart late apoptosis caused by M protein, which would facilitate the viral release and dissemination.

c. NV suppresses TNFα-mediated NF-kB activation

Nuclear factor-kB (NF-kB) plays a pivotal role in immediate early immune response to pathogens, in promoting inflammation and in regulation of cell survival, and its activation is a key step in TNFα-mediated immunomodulatory pathways. NF-kB exists as an inactive cytoplasmic form by binding to its inhibitory protein, IkBα. Upon stimulation, IkBα is phosphorylated by kinases and subsequently degraded by the proteasome, which allows nuclear translocation of NF-kB and activation of NF-kB target genes including proinflammatory and antiviral cytokine genes such as tumor necrosis factor *(tnf)-α*, among others (e. g.: *il1b and il8*) [254]. On the other hand, TNF-α is a proinflammatory cytokine produced by immune cells in response to pathogens that can induce either NF-kB mediated survival or apoptosis, depending on the cellular context [280], and whose antiviral activity has been well demonstrated in various viruses in mammals [254]. However, the role of TNF-α in fish viral infection still remains uncertain despite the fact that Roca et al. (2008) reported that this cytokine facilitated *in vitro* replication and increased *in vivo* susceptibility to spring viremia of carp virus (SVCV) [255].

In our study, we found downregulated by rNV most of members of the tumor necrosis factor (*tnf*) family, which promote inflammation, including tumor necrosis factor alpha (*tnfα*) that is one of the most important inducers of systemic inflammation **(Figure 5, Paper I)**. Moreover, we found downregulated the p100/p52 transcription factor (*nf-kb2*) **(Figure 4, Paper I)**. These findings altogether support the results obtained by Kim and Kim (2013), who suggested that NV of VHSV suppresses TNF-α-mediated NF-κB activation *in vitro* [254]. Furthermore, they generated a NV gene knock-out recombinant VHSV (rVHSV-ΔNV-EGFP), which induced significantly higher NF-κB activity in EPC cells than wild-type VHSV, suggesting that VHSV adopted a strategy to suppress early activation of NF-κB in host cells through the NV gene [254].

2. Novel effects of VHSV NV

The immune system is a set of cellular and humoral components to defend the body against foreign microorganisms, among other threats. As in mammals, the fish immune system is also divided into innate and adaptive, both in turn divided into cell mediated defense and humoral factors, although today it is known that these two systems work together to trigger defense processes [265].

The innate system includes all components present in the body before the appearance of the pathologic agent, as the first line of defense that acts faster than the adaptive, and is able to recognize conserved molecular structures common to pathogenic microorganisms (PAMPs) such as viral RNA molecules, among others, through their interaction with specific receptors (TLRs). These mechanisms of recognition may lead directly to successful removal of pathogens, for instance by phagocytosis, or may trigger additional protective responses through induction of adaptive immune responses. Genes of the innate immune system have a diverse array of functions. For example, some genes promote phagocytosis (e.g.: TLR-inducible genes), and others, stimulate and help guide the migration of cells and further direct the immune response (e.g.: cytokines and chemokines) [265].

In this work, we not only confirmed previous results regarding TNF-α-mediated NF-kB activation, *mx*, the downregulation of some interferons and the anti-apoptotic role of NV but also described a plethora of new immune innate and adaptive response genes differently expressed compared to the polyH control [257] **(Paper I, and Table 5)**. Among these genes, we found novel and numerous innate genes expressed downwards such as interferons, tumor necrosis factors, toll-like receptors, interleukins, chemokines and transcription factors **(Table 5).** However, a few genes were found upregulated, and in some cases these genes are inhibitors of inflammation (such as *il20* and *il10*), favouring the shut-off of the immune response. Moreover, downregulation of TLR genes by NV showed that immune response is also hampered at recognition level.

Among transcription factor genes, we detected some of them downregulated such as *stat1*, *jun*, *atf1*, *stat3*, *stat5*, *tbx21*, *p100*, *cebp*, *srcap*. STAT factors play a key role in important pathways promoting interferon expression (JAK/STAT) or regulating other signaling pathways (*stat3*, *stat5*). JUN and ATF1 form a dimer to give rise AP1, which targets DNA response elements leading to many immunological effects including those related to *tnf* and *tnf*-related genes. NV downregulated NF-kB subunit p100 (p52) and this fact seriously affects proliferation and immune response targets at nucleus. In addition, there is an accumulative effect because CD40 (*tnf5 gene*) is also downregulated diminishing p100 processing to p52 [281]. *Cebp* gene, also known as nuclear factor of interleukin 6, is involved in immune and inflammatory responses, among other processes. Tbx21 protein is a Th1 cell-specific transcription factor that controls the expression of the hallmark Th1 cytokine, interferon-gamma (IFNg). *Srcap* codes for a helicase that acts as a coactivator for CREB-mediated transcription, steroid receptor-mediated transcription, and Notch-mediated transcription. These transcription factors affect the low expression of immune response genes that cannot close the regulatory circle (i. e.: IFN-STAT1-IFN) or regulate other pathways appropriately.

On the other hand, the adaptive system recognizes foreign structures by means of two cellular receptors (BCR and TCR) and is highly regulated by several mechanisms. Adaptive immunity generally starts days after infection and is capable of recognizing specific protein motifs of peptides, which leads to a response that increases in both speed and magnitude with each successive antigen exposure and produces immunological memory, the basis of vaccine development. The main effector cells of the adaptive immune response are the lymphocytes, specifically B and T cells. When B cells are activated, they are capable of differentiating into plasma cells that can secrete antibodies. Upon activation T cells differentiate into either helper T cells or cytotoxic T cells. Helper T cells are capable of activating other cells of the adaptive immune response such as B cells and macrophages, while cytotoxic T cells upon activation are able to kill cells that have been infected [282]. NV induced the downregulation (and certain upregulation) of humoral and cellular adaptive immune response genes such as the antigen presentation machinery, cluster differentiation antigens and some interleukins and chemokines, macrophage-related genes and the classic complement pathway **(Table 5).** Regarding humoral adaptive genes we found downregulated *il2* and *ifng* among others. Within cellular adaptive genes those found downregulated in trout are *cd79a* (forms heterodimer with BCR), antigen processing and presentation molecules (*tap*, *lmp2*, *tapbp*), *cd2* (partipates in adhesion T-lymphocytes and APC), *cd3* (is part of TcR), and *cd28* (co-stimulatory molecule in antigenic presentation). Moreover, NV also downregulated many genes which connect innate with adaptive immunity such as type I *ifn*, *tnfa*, *il12*, *il15*, and *il1b*.

The vast downregulation (and certain upregulation) of novel innate immune-related genes and adaptive immune-related genes induced by rNV **(Table 5)** suggests that NV impairs the normal development of the host innate immune response and also delays the start of adaptive responses, interfering with the antiviral state and inflammatory response of host cells and enhancing viral replication. All these inhibitory effects disappear when using recombinant viruses in different fish models. So, *in vivo* protection has been reported for VHSV challenge with NV knockout VHSV in olive flounder [42] and for IHNV challenge with NV knockout [240] and NV-ΔEGDL [248] IHNV for trout as live-attenuated vaccine candidates in a dose dependent manner.

Table 5. Novel modulation (downregulation or upregulation) of statistically significant immune-related genes induced by the recombinant NV protein (rNV) of VHSV and the possible effects of this gene modulation on host immune response. Interesting immune-related genes downregulated or upregulated in this study that were not statistically significant are included and marked in red **(Figures 4 to 7, Paper I).**

NOVEL IMMUNE GENE MODULATION INDUCED BY NV (VHSV)		
REGULATION	***GENES***	***EFFECTS ON IMMUNE SYSTEM***
Downregulation	***IFN and IFN-related*** *(ifn1, ifng, irf1, irf2, irf10)*	Interference with the host **antiviral state** enhancing viral replication
	VHSV-induced genes (vigs)	Suppression of early host responses
	Caspases (Casp) *(**Casp1a**, **Casp6**, Casp9)*	Inhibition of early **apoptosis** favouring viral replication
	Transcription factors (TF) *(Stat1, jun, atf1, **stat3, stat5, tbx21, p100, cebp** and **srcap**)*	Low expression of proteins related to immune system
	TNF-related *(tnfa, tnf5 or CD40, tnf10, tnf11, tnf13, tnfr and ltb2)*	Inhibition of **inflammatory responses**
	TLR-related *(tlr1, tlr3, tlr7 and tollip1 and 2)*	Inhibition of viral **RNA detection**
	APM genes *(tapbp, tap1 and 2, lmp2 and b2m)*	Impairment of **appropriate processing of pathogen antigens**, delaying the adaptive immune response
	CD antigens *(cd2, cd4, **cd8**, **cd28**, cd3e, cd79a and cd200)*	**Adaptive immune response** delay, except for cd200 (NK immunosuppression)
	IL-related *(**il1b**, il2, **il8**, il15, il16, il17d, il29 and ilr3)*	Downregulation of *nfkb* and therefore interference of the **immune response** in many other pathways. Inhibition of **inflammatory response.**
	MA-related genes *(cd11, pbef, cdh, mcsf and **nramp**)*	Interference with **phagocytosis, apoptosis and antigen processing and presentation.**
	Chemokine (CK) genes *(ck2,ck6, **ck10**, **ck12**, socs, cc, cxc, cxcd1 and 2)*	Interference with **inflammation and** correct triggering of the **systemic immune response**
	Complement (CO)-genes *(prf, c1q, cr1 and c4)*	Delay **antibody and/or cellular-mediated responses**
Upregulation	**CD antigens** *(cd33, cd97, cd209)*	Stimulation of the **host immune response** like any other protein antigen
	IL-related genes *(**il17a** and **il17c**, il10, il20)*	Inhibition of **inflammatory responses**
	MA-related genes *(csf1)*	Interference with **phagocytosis, apoptosis and antigen processing and presentation.**
	Chemokine (CK) genes *(ck11, ckb14, ccr5 and cxcr)*	Interference with **inflammation and** correct triggering of the **systemic immune response**
	Complement (CO)-genes *(c3a, c3.3, cfi, **h1**)*	Delay **antibody and/or cellular-mediated responses** and also possible host compensatory mechanisms

The novel data presented in this work **(Paper I)** [257] improve our knowledge of the immunosuppression mechanisms used by the NV protein of VHSV and could explain much better than before the protection induced by NV knockout novirhabdoviruses as due to the lack of an extensive inhibition of host early immune gene responses.

3. Multipath genes reveal possible VHSV NV targets

The potential of multipath genes to suggest functional studies had been used previously to investigate the effects of spring viraemia virus (SVCV) infection on zebrafish and suggest preventive drug candidates [283]. This analysis is an approach to detect genes involved in the immune response in the multiple and complex existing pathways, helping to establish close relationships among genes and possible targets. To test for differentially expressed multipath genes, microarray genes were first classified into 74 rainbow trout immune-related pathways. In order to do that, the list of accession numbers of the microarray genes was first compared with the rainbow trout accession numbers obtained by manually searching their corresponding orthology in immune-related human pathways actually present in public databases. The classified genes were then systematically searched for genes (i) common to at least six immune-related pathways [283], (ii) with differential expression folds <0.66 or >1.5 and (iii) significantly different from one of the 0.66/1.5 thresholds ($p \leq 0.05$).

Results obtained in the multipath analysis showed 14 genes **(Table 1, Paper I)** that accomplished the selection criteria. *Stat1* is the multipath gene more downregulated that participates in more pathways (n=27), followed in the number of pathways by *jun* and *atf1*. Other important downregulated gene is *il1b* although is participating in 8 immune pathways. *Stat1* participates in important pathways such as chemokine signaling pathway, JAK-STAT signaling pathway, and Toll-like receptor signaling pathway (see all in https://www.genome.jp/dbget-bin/www_bget?ko:K11220). *Jun* and *atf1* participate as transcription factors in many pathways (see all in https://www.genome.jp/dbget-bin/www_bget?hsa:3725 and https://www.genome.jp/dbget-bin/www_bget?ko:K09053, respectively) leading to a reduced activity in gene transcription. Moreover, other genes detected suggest that apoptosis (*casp6*), interferon (*ifn1*, *irf1*, *irf10*, *ifng*) and tumor necrosis factor receptors (*traf2*, *traf3*) are implied in immune pathways affected by NV. Other genes common to more than 6 pathways were downregulated in microarray but not differentially expressed, for instance, *p53*, *tnf6*, *il6*, *mhc2.daa*, *junb*, *if2a*, *myc*, *tgfb*, *il12b*, *il8*, *grb and irak4*. Altogether, these data showed the wide downregulatory effects induced by NV and could led to establish relationships among genes and pathways in the immune response against NV and to help searching initiators in signaling cascade.

D. VHSV INFECTION AND THE MEASUREMENT OF NEUTRALIZING ANTIBODIES

With the development of reverse genetics technology, numerous NV-deleted recombinant viruses have been obtained and assayed (see **Table 4**) observing that their pathogenicity *in vivo* is lower, reason why they have been considered to be used as live attenuated vaccines. This is partly due to the absence of negative effects in the host caused by the NV protein (absent in these recombinant viruses). The studies with these viruses have been carried out in different models of fish: zebrafish, rainbow trout, yellow perch and olive flounder **(Table 4).** In each study, fish survival was assessed after vaccination and further challenge, displaying a high survival of the fish treated with the recombinant viruses independently of the fish model.

Surprisingly, in these studies, humoral immune parameters such as neutralizing antibodies against virus measured by seroneutralization assays have not been established yet. Neutralizing antibodies are directed against viral surface glycoproteins (G) and are an important component of the protective immune response of rainbow trout to VHSV [211]. This set of antibodies is measured by indirect diagnostic assays such as neutralization tests [284], among others (ELISA, etc). On most sampling occasions, antibodies were detected even when the virus was not, suggesting that these fish have survived a relatively recent VHSV infection. To date, however, studies on neutralizing antibodies have been infrequent due to lack of availability of reliable serological diagnostic tests. Consequently, it has been difficult to ascertain the proportion of wild fish that have been infected by VHSV and have survived. It has also been difficult to infer whether the virus persists in wild fish populations in postepizootic years.

The standard techniques used normally require relatively large volumes of plasma (or serum) that are sometimes difficult to obtain. The work presented as part of this thesis **(Paper III)** report a seroneutralization technique that required minimum volumes of plasma (or serum) from fish and a semi-automated method for neutralizing antibody screening. These results could serve as a basis for future studies on vaccine effectiveness that want to delve into the antibody response to VHSV independently of the model used. These data would be important to correlate the presence of neutralizing antibodies with fish survival and the virus and virus-dependent dose used in the vaccine, as well as the long-term protection. Moreover, the incorporation of serological testing into current VHSV surveillance programmes would be useful to determine the immune status of populations and also to provide a more thorough understanding of virus distribution.

V. CONCLUSIONES

CONCLUSIONS

CONCLUSIONES

1. La proteína VHSV NV 07.71 puede ser expresada en sistemas procariotas y posteriormente purificada para ser inoculada en truchas. Sin embargo, la NV tiende a precipitar y sólo una pequeña parte que permanece soluble puede ser utilizada para este fin.

2. Por primera vez, se ha caracterizado en trucha el perfil transcriptómico de genes de respuesta inmune inducido por la NV a 48 horas post-infección (hpi).

3. Algunos de los efectos de la NV observados en trucha confirmaron los efectos descritos previamente como son la regulación negativa de *mx*, *ifn1* and caspasa 9 (*casp9*).

4. Se han caracterizado en el transcriptoma de tejidos inmunitarios de trucha gran número de cambios nuevos inducidos por la NV en genes de respuesta inmune innata y adaptativa, la mayoría de ellos regulados negativamente.

5. Entre los nuevos genes downregulados por la NV se encuentran genes de respuesta inmune innata relacionados con interferón (*iip30*, *ipf35*, *iip2*, *gig2*, *irf7*, *irf10*), factores de transcripción (*jun*, *atf-1, stat1, stat3*), factores de necrosis tumoral (*tnfa*, *traf3*) y sus receptores, receptores tipo Toll, interleuquinas, citoquinas, genes CD, complemento y otros genes co-estimuladores.

6. Algunos genes de respuesta inmune adaptativa también estaban downregulados por la proteína NV, como son los genes del sistema principal de histocompatibilidad de clase II, y los de procesamiento y presentación antigénica (*tap*, *lmp*, *b2m*).

7. En conjunto, estos datos mostraron que la NV induce una importante disminución de la expresión génica que no sólo lleva a un estado antiviral deficiente sino que también conlleva una comunicación ineficaz entre las respuestas inmunes innata y adaptativa.

8. Las células de pez transfectadas con la NV de VHSV, IHNV e HIRRV presentan un efecto inhibitorio de la expresión génica de *mx,* estando las NVs implicadas en la patogenicidad. Estos son datos confirmatorios para VHSV e IHNV pero nuevos para HIRRV. Además, la NV de SHRV no produjo la downregulación de *mx* sugiriendo que no es necesaria para la replicación del virus snakehead.

9. Las variantes NVD36A, NVR39A y NVD41A aumentaron su capacidad inhibitoria sobre los transcritos de *mx* e *il8* en comparación con la NV nativa. Estas variantes podrían ser más estables e interaccionar de forma más eficiente con su ligando, la fosfatasa PPM1Bb, la cual podría defosforilar en mayor medida TBK1 y, en consecuencia, conducir a mayor inhibición de las vías de señalización descendentes.

10. Se demuestra que la región bisagra de la NV de VHSV puede afectar a su función y que no contiene una señal de localización nuclear (NLS) como la definida para la NV de IHNV.

11. Se ha mapeado la funcionalidad de la NV y ésta depende de la parte N-terminal de la NV (F1) y no así de la parte C-terminal (F2), aunque esta última parte podría conferir mayor estabilidad a la estructura terciaria de la proteína.

12. La proteína NV nativa, y todas las variantes y fragmentos funcionales de la misma, se localizan en el citoplasma, mientras que el fragmento no funcional (F2, C-terminal) se localiza de manera difusa tanto en el citoplasma como en el núcleo. Así, existe una clara correlación entre el patrón de localización y la función.

13. El desarrollo de una técnica de detección semi-automática de anticuerpos neutralizantes frente a VHSV en pequeños volúmenes de plasma permitiría su aplicación al estudio de la efectividad de las vacunas frente al virus, particularmente aquellas basadas en la deleción de la NV.

CONCLUSIONS

1. The VHSV NV 07.71 protein can be expressed in prokaryotic systems and further purified to be injected in trout. However, NV tends to precipitate and only a small soluble amount can be used for this purpose.

2. For the first time, it has been characterized in trout the immune transcriptome profile induced by NV at 48 hours post-infection (hpi).

3. Some of the observed NV effects in trout confirmed those previously described such as: downregulation of *mx*, *ifn1* and caspase 9 (*casp9*).

4. The major immune-related transcriptome changes induced by NV are a plethora of new affected genes, mostly downregulated, from innate and adaptive immune response in trout.

5. Among these new downregulated genes by NV are found innate immune response genes: interferon-related genes (*iip30*, *ipf35*, *iip2*, *gig2*, *irf7*, *irf10*), transcription factors (*jun*, *atf-1, stat1, stat3*), tumor necrosis factors (*tnfa*, *traf3*) and their receptors, Toll-like receptors, interleukins, cytokines, CD (cluster of differentiation), complement, and other co-stimulatory genes.

6. Adaptive immune response genes are also downregulated by NV protein: major histocompatibility complex class II genes, and antigen processing and presenting genes (*tap*, *lmp*, *b2m*).

7. Overall, these data showed that NV induces an important gene shutdown not only leading to a weak antiviral state but also to a dysfunctional crosstalk between innate and adaptive immune response.

8. Fish cells transfected with NV from VHSV, IHNV, and HIRRV exhibit an inhibitory effect of gene expression, downregulating *mx* among others and being NVs implied in pathogenicity. These are confirmatory data for VHSV and INHV but new for HIRRV. Moreover, NV from SHRV did not downregulate *mx* supporting that it is not necessary for snakehead virus replication.

9. Variants NVD36A, NVR39A and NVD41A improved their ability to downregulate *mx* and *il8* transcripts compared to wt-NV. These variants could have acquired more stability and improved interaction with its target the phosphatase PPM1Bb, which would dephosphorylate TBK1 in higher degree and subsequently would lead to stronger inhibition of downstream pathways.

10. It is demonstrated that the hinge region of the VHSV NV can affect its function and that it does not contain a nuclear localization signal (NLS) as defined for the IHNV NV.

11. NV functionality has been mapped out, and this rely on N-terminal part of NV (F1) rather than the C-terminal one (F2), even if this latter sequence could confer higher molecular stability to the tertiary structure of the protein.

12. Wild-type NV, and all NV variants and functional NV fragments, are located in cytoplasm with the same pattern, whereas non-functional NV fragment (F2, C-terminal) is diffusely located in both cytoplasm and nucleus. Thus, there is a clear correlation between the location pattern and the function.

13. The development of a semi-automatic assay to detect anti-VHSV neutralizing antibodies in low volumes of plasma blood samples could allow its application in future studies on vaccine effectiveness, particularly those based on NV deletion.

VI. REFERENCES

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